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<p>(54) Title: FRAMESHIFT MUTANTS OF BETA-AMYLOID PRECURSOR PROTEIN AND UBIQUITIN-B AND THEIR USE</p> <p>(57) Abstract</p> <p>Frameshift Mutants β-Amyloid precursor peptides and mutant ubiquitin-B associated with Alzheimer's disease and Down syndrome eliciting T cellular immunity for use in compositions for the treatment and/or prophylaxis of Alzheimer's disease and/or Down syndrome.</p>			

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**FRAMESHIFT MUTANTS OF BETA-AMYLOID PRECURSOR PROTEIN AND
UBIQUITIN-B AND THEIR USE**

5 The present invention relates to peptides for treatment and/or prophylaxis of Alzheimer's disease and Down syndrome.

Alzheimer's disease and treatment of Down syndrome are both
10 associated with frameshift mutations occurring at the transcriptional level or by posttranscriptional editing of RNA during the encoding of β-Amyloid precursor protein (βAPP) and ubiquitin-B (Ubi-B). Such frameshift mutations give rise to mutant βAPP and Ubi-B protein products which
15 are characterised by aberrant protein sequences at the carboxyl terminus. Peptides covering, either completely or parts of, the aberrant parts of mutant βAPP or Ubi-B protein products elicit T cellular immunity and can therefore be useful in compositions for the treatment of
20 Alzheimer's disease and Down syndrome. Further the peptides of this invention can be used as a prophylactic anti-Alzheimer's disease vaccine.

The invention also relates to DNA sequences encoding
25 peptides corresponding to aberrant βAPP and Ubi-B protein sequences found in Alzheimer's disease and Down syndrome patients, and to vectors comprising at least one insertion site containing a DNA sequence encoding at least one such peptide.

30 Further the invention relates to methods for the treatment and/or prophylaxis of Alzheimer's disease by administration of at least one mutant βAPP and/or Ubi-B peptide or a recombinant virus vector comprising at least one insertion

site containing a DNA sequence encoding at least one mutant β APP and/or one mutant Ubi-B peptide.

The present invention represents a development of a
5 treatment and/or prophylaxis for Alzheimer's disease based
on the use of peptides to generate activation of the T
cellular arm of the body's own immune system against cells
producing mutant β APP and Ubi-B protein products associated
with Alzheimer's disease.

10

The development and use of the methods for treatment of
Alzheimer's disease may also be directly applicable for
treatment of patients with Down syndrome.

15

Technical Background

Peptides corresponding to aberrant protein sequences
resulting from frameshift mutations in genes in cancer
20 cells elicit specific T cellular immunity and can be used
as anti-cancer vaccines (ref. Norwegian patent applications
filed at the same date as the present application by Norsk
Hydro ASA). In the same manner peptides corresponding to
aberrant protein sequences resulting from frameshift
25 mutations associated with other diseases can be used to
develop treatments of that diseases based on generation of
specific T cellular immunity.

Frameshift mutations result in completely new amino acid
30 sequences in the C-terminal part of the proteins,
prematurely terminating where a novel stop codon appears.
This results in two important consequences:

35 1) The truncated protein resulting from the frameshift is
generally nonfunctional, in most cases resulting in
"knocking out" of an important cellular function. Aberrant

proteins may also gain new functions such as the capacity to aggregate and form plaques. In both cases the frameshift results in disease.

5 2) The aberrant new C-terminal amino acid sequence resulting from the frameshift is foreign to the body. It does not exist prior to the mutation, and it only exists in cells having the mutation.

10 Since the mutant part of the proteins proteins are completely novel and therefore foreign to the immune system of the carrier, they may be recognized by T-cells in the repertoire of the carrier. So far, nobody has focused on this aspect of frameshift mutations, and no reports exist
15 on the characterization of frameshift peptides from coding regions of proteins as antigens. This concept is therefore novel and forms the basis for developing vaccines based on these sequences. It follows that such vaccines may also be used prophylactically in persons who inherit defective
20 genes or in other ways are disposed for frameshift mutations. Such vaccines will therefore fill an empty space in the therapeutic armament against inherited forms of disease.

25 It has been shown that single amino acid substitutions in intracellular "self"-proteins may give rise to tumour rejection antigens, consisting of peptides differing in their amino acid sequence from the normal peptide. The T cells which recognise these peptides in the context of the
30 major histocompatibility (MHC) molecules on the surface of the tumour cells, are capable of killing the tumour cells and thus rejecting the tumor from the host.

35 In contrast to antibodies produced by the B cells, which typically recognise a free antigen in its native conformation and further potentially recognise almost any

site exposed on the antigen surface, T cells recognise an antigen only if the antigen is bound and presented by a MHC molecule. Usually this binding will take place only after appropriate antigen processing, which comprises a proteolytic fragmentation of the protein, so that the resulting peptide fragment fits into the groove of the MHC molecule. Thereby T cells are enabled to also recognise peptides derived from intracellular proteins. T cells can thus recognise aberrant peptides derived from anywhere in the cells, in the context of MHC molecules on the surface of the cells, and can subsequently be activated to eliminate the cells harbouring the aberrant proteins.

M.Barinaga, Science, 257, 880-881, 1992 offers a short review of how MHC binds peptides. A more comprehensive explanation of the Technical Background for this Invention may be found in D. Male et al, Advanced Immunology, 1987, J.B.lippincott Company, Philadelphia. Both references are hereby included in their entirety.

The MHC molecules in humans are normally referred to as HLA (human leukocyte antigen) molecules. They are encoded by the HLA region on the human chromosome No 6.

The HLA molecules appear as two distinct classes depending on which region of the chromosome they are encoded by and which T cell subpopulations they interact with and thereby activate primarily. The class I molecules are encoded by the HLA A, B and C subloci and they primarily activate CD8+ cytotoxic T cells. The HLA class II molecules are encoded by the DR, DP and DQ subloci and primarily activate CD4+ T cells, both helper cells and cytotoxic cells.

Normally every individual has six HLA Class I molecules, usually two from each of the three groups A,B and C. Correspondingly, all individuals have their own selection

of HLA Class II molecules, again two from each of the three groups DP, DQ and DR. Each of the groups A, B, C and DP, DQ and DR are again divided into several subgroups. In some cases the number of different HLA Class I or II molecules
5 is reduced due to the overlap of two HLA subgroups.

All the gene products are highly polymorphic. Different individuals thus express distinct HLA molecules that differ from those of other individuals. This is the basis for the
10 difficulties in finding HLA matched organ donors in transplantations. The significance of the genetic variation of the HLA molecules in immunobiology is reflected by their role as immune-response genes. Through their peptide binding capacity, the presence or absence of certain HLA
15 molecules governs the capacity of an individual to respond to peptide epitopes. As a consequence, HLA molecules determine resistance or susceptibility to disease.

T cells may control the development and growth of cells
20 producing aberrant proteins by a variety of mechanisms. Cytotoxic T cells, both HLA class I restricted CD8+ and HLA Class II restricted CD4+, may directly kill cells carrying the appropriate antigens. CD4+ helper T cells are needed for cytotoxic CD8+ T cell responses as well as for antibody
25 responses, and for inducing macrophage and LAK cell killing.

A requirement for both HLA class I and II binding is that the peptides must contain a binding motif, which usually is
30 different for different HLA groups and subgroups. A binding motif is characterised by the requirement for amino acids of a certain type, for instance the ones carrying large and hydrophobic or positively charged side groups, in definite positions of the peptide so that a narrow fit with the
35 pockets of the HLA binding groove is achieved. The result of this, taken together with the peptide length restriction

of 8-10 amino acids within the binding groove, is that it is quite unlikely that a peptide binding to one type of HLA class I molecules will also bind to another type. Thus, for example, it may very well be that the peptide binding motif 5 for the HLA-A1 and HLA-A2 subgroups, which both belong to the class I gender, are as different as the motifs for the HLA-A1 and HLA-B1 molecules.

For the same reasons it is not likely that exactly the same 10 sequence of amino acids will be located in the binding groove of the different class II molecules. In the case of HLA class II molecules the binding sequences of peptides may be longer, and it has been found that they usually contain from 10 to 16 amino acids, some of which, at one or 15 both terminals, are not a part of the binding motif for the HLA groove.

However, an overlap of the different peptide binding motifs of several HLA class I and class II molecules may occur. 20 Peptides that have an overlap in the binding sequences for at least two different HLA molecules are said to contain "nested T cell epitopes". The various epitopes contained in a "nested epitope peptide" may be formed by processing of the peptide by antigen presenting cells and thereafter be 25 presented to T cells bound to different HLA molecules. The individual variety of HLA molecules in humans makes peptides containing nested epitopes more useful as general vaccines than peptides that are only capable of binding to one type of HLA molecule.

30 Effective vaccination of an individual can only be achieved if at least one type of HLA class I and/or II molecule in the patient can bind a vaccine peptide either in its full length or as processed and trimmed by the patient's own 35 antigen presenting cells.

The usefulness of a peptide as a general vaccine for the majority of the population increases with the number of different HLA molecules it can bind to, either in its full length or after processing by antigen presenting cells.

5

In order to use peptides derived from an aberrant protein resulting from mutational events in cells as vaccines or therapeutic agents to generate CD4+ and/or CD8+ T cells, it is necessary to investigate the mutant protein in question and identify peptides that are capable, eventually after processing to shorter peptides by the antigen presenting cells, to stimulate T cells.

10

15 Definition of Problem solved by the Invention.

At present no drug or other treatment is able to eliminate the cells producing the aberrant proteins responsible for the degenerative process in the brain of patients afflicted 20 with Alzheimers disease. There is a continuing need for treatment and prophylaxis of Alzheimer's disease. The present invention will contribute to supply new peptides that can have use as treatment and prophylaxis of Alzheimer's disease. Our approach is aimed at directly 25 eliminating the cells responsible for the degenerative process.

The peptides may also be applicable for treatment of patients with Down syndrome.

30

Definition of the Invention

A main object of the invention is to obtain peptides 35 corresponding to peptide fragments of aberrant β APP and

Ubi-B proteins found in Alzheimer's disease and/or Down syndrome patients which can be used to stimulate T cells.

Another main object of the invention is to develop a treatment for Alzheimer's disease based on the T cell immunity which may be induced in patients by stimulating their T cells either *in vivo* or *ex vivo* with the peptides according to the invention.

A third main object of the invention is to develop a vaccine to prevent the establishment of Alzheimer's disease based solely or partly on peptides corresponding to peptides of the present invention which can be used to generate and activate T cells which produce cytotoxic T cell immunity against cells producing the mutant β APP and mutant Ubi-B proteins.

A fourth main object of the invention is to design a treatment or prophylaxis for Alzheimer's disease specifically adapted to a human individual in need of such treatment or prophylaxis, which comprises administering at least one peptide according to this invention.

A fifth object of the invention is to obtain a treatment for patients with Down syndrome using the same methods as for the treatment of Alzheimer's disease.

These and other objects of the invention are achieved by the attached claims.

Frameshift mutations can occur at the gene level, transcriptional level or by posttranscriptional editing of RNA and result in premature stop codons and therefore a deletion of sometimes large parts of the proteins. Aberrant proteins arising from frameshift mutations have generally

not been considered to be immunogenic and have therefore
not been considered as targets for immunotherapy. Thus it
has now surprisingly been found that a group of new
peptides corresponding to aberrant proteins resulting from
5 frameshift mutations associated with Alzheimer's disease
and Down syndrome are useful for eliciting T cell responses
against cells producing such aberrant proteins.

Genes containing a mono nucleoside base repeat sequence,
10 for example of deoxyadenosine bases, or a di-nucleoside
base repeat sequence, for example of
deoxycytosine-deoxythymidine units, are susceptible to
frameshift mutations. The frameshift mutations occur,
respectively, either by insertion of one or two of the
15 mono-nucleoside base residue or of one or two of the
di-nucleoside base unit in the repeat sequence, or by
deletion of one or two of the mono-nucleoside base residue
or of one or two of the di-nucleoside base unit from the
repeat sequence. A frameshift mutation will from the point
20 of mutation encode a protein with a new and totally
different amino acid sequence as compared to the normal
protein. This mutant protein with the new amino acid
sequence at the carboxy end will be specific for all cells
in which such frameshift mutations have occurred.

25 In the remainder of this specification and claims the
denomination frameshift mutant peptides will comprise such
proteins and peptide fragments thereof.

30 These peptides are at least 8 amino acids long and
correspond to frameshift mutant β APP and/or Ubi-B protein
sequences associated with Alzheimer's disease and/or Down
syndrome.

A peptide according to this invention is characterised in that it

5 a) is at least 8 amino acids long and is a fragment of a mutant β APP and/or Ubi-B protein arising from a frameshift mutation associated with Alzheimer's disease or Down syndrome;

and

10

b) consists of at least one amino acid of the mutant part of the mutant β APP and/or Ubi-B protein;

and

15

c) comprises 0-10 amino acids corresponding to the carboxyl terminus of the normal part of the protein sequence preceding the amino terminus of the mutant sequence and may further extend to the carboxyl terminus of the mutant part 20 of the protein as determined by a new stop codon generated by the relevant frameshift mutation;

and

25 d) induces, either in its full length or after processing by antigen presenting cells, T cell responses.

The peptides of this invention contain preferably 8-25, 30 9-20, 9-16, 8-12 or 20-25 amino acids. They may for instance contain 9, 12, 13, 16 or 21 amino acids.

It is most preferred that the peptides of the present invention are at least 9 amino acids long, for instance

9-18 amino acids long, but due to the processing possibility of the antigen presenting cells also longer peptides are very suitable for the present invention. Thus the whole mutant amino acid sequence may be used as a

5 frameshift mutant peptide according to the present invention, if it comprises 8 amino acids or more.

The invention further relates to a method for vaccination of a person disposed for Alzheimer's disease, consisting of administering at least one peptide of the invention one or 10 more times in an amount sufficient for induction of T-cell immunity to the mutant β APP and/or Ubi-B proteins.

The invention also relates to a method for treatment of a patient with Alzheimer's disease, consisting of

15 administering at least one peptide of the invention one or more times in an amount sufficient for induction of T-cell immunity to the mutant β APP and/or Ubi-B proteins.

The invention also relates to a method for treatment of a 20 patient with Down syndrome, consisting of administering at least one peptide of the invention one or more times in an amount sufficient for induction of T-cell immunity to the mutant β APP and/or Ubi-B proteins.

25

Detailed Description of the invention.

In the present description and claims, the amino acids are represented by their one letter abbreviation as known in 30 the art.

The peptides of the present invention are exemplified by the β APP and Ubi-B frameshift mutations associated with Alzheimer's disease and Down syndrome:

In Alzheimer's and Down syndrome patients, intracellular and extracellular depositories of proteins in tangles, neurophil threads and neuritic plaques are correlated with neuronal dysfunction leading to dementia (R.D.Terry et al 5 in *Alzheimer Disease*, R.D.Terry, R.Katzman, K.L.Bick, Eds. (Raven, New York, 1994) pp. 179-196). These protein deposits have been shown to contain forms of β amyloid precursor protein (β APP) and ubiquitin-B (Ubi-B) that are aberrant in the carboxyl terminus, and it has further been 10 shown that these aberrant protein sequences are results of frameshift mutations which probably occur at the transcriptional level or by posttranscriptional editing of RNA (F.W. van Leeuwen et al, *Science*, vol 279, pp. 242-247).

15 In the case of β APP two frameshift mutations have been observed, one by deletion of the di-nucleoside deoxyguanosine-deoxyadenosine (GA) unit from the (ACC)GAGAGA(ATG) sequence in exon 9, and one by deletion of a GA unit from the (CAT)GAGAGA(ATG) sequence in exon 10. 20 The mutant β APP peptides resulting from these frameshift mutations are shown in table 1. The peptides with seq id nos 1 and 4 are the mutant part of the β APP protein sequence and the peptides with seq id nos 2, 3 and 5 represent mutant peptides extended into the normal β APP 25 sequence at the amino terminus.

normal β APP; RLEAKHRERMSQVMREWEAERQAKNLPK
seq id no 1; NVPGH~~E~~RMGRGRTSSKELA
seq id no 2; RLEAKHRENVPGHERMGRGRTSSKELA
30 seq id no 3; RLEAKHRENVPGHERMG
seq id no 4; MGRGRTSSKELA
seq id no 5; ERMSQVMRMGRGRTS

Table 1.

Also in the case of Ubi-B two frameshift mutations have been observed, one by deletion of the di-nucleoside deoxyguanosine-deoxythymidine (GT) unit from the (TCT)GAGAGGT(GGT) sequence in exon, and one by deletion of 5 a di-nucleoside deoxycytosine-deoxythymidine (CT) unit from the (TCA)CTCT(GGA) sequence in exon 3. The mutant Ubi-B peptides resulting from these frameshift mutations are shown in table 2. The peptides with seq id nos 6 and 9 are the mutant part of the Ubi-B protein sequence and the 10 peptides with seq id nos 7, 8 and 10 represent mutant peptides extended into the normal Ubi-B sequence at the amino terminus.

15

normal Ubi-B;	HLVLRLRGGMQIFVKTLTGKTITLEVEPSD
seq id no 6;	YADLREDPDRQDHHPGSGAQ
seq id no 7;	HLVLRLRGYADLREDPDRQDHHPGSGAQ
seq id no 8;	HLVLRLRGYADLREDPD
20 seq id no 9;	GGGAQ
seq id no 10;	TLTGKTITGGGAQ

Table 2.

25

The mutant β APP and Ubi-B proteins are only encoded for by cells in which corresponding frameshift mutations have occurred and are therefore targets for specific immunotherapy of Alzheimer's disease and Down syndrome.

30

According to the present invention, peptides corresponding to mutant β APP and mutant Ubi-B proteins can be used to elicit T cellular immunity and specific killing of cells producing mutant β APP and mutant Ubi-B proteins, which in 35 Alzheimer's disease and Down syndrome patients are correlated with neuronal dysfunction leading to dementia.

Other peptides of the invention can be fragments of the peptides listed in the Tables 1 and 2 above. Such fragments are most preferred from 9-16 amino acids long and include
5 at least one amino acid from the mutant part of the protein.

As used in this description and claims the term fragment is intended to specify a shorter part of a longer peptide or
10 of a protein.

15 Synthesis

The peptides are synthesised by using continuous flow solid phase peptide synthesis. N-a-Fmoc-amino acids with appropriate side chain protection are used. The Fmoc-amino
20 acids are activated for coupling as pentafluorophenyl esters or by using either TBTU or di-isopropyl carbodi-imide activation prior to coupling. 20% piperidine in DMF is used for selective removal of Fmoc after each coupling. Cleavage from the resin and final removal of side
25 chain protection is performed by 95% TFA containing appropriate scavengers. The peptides are purified and analysed by reversed phase (C18) HPLC. The identity of the peptides is confirmed by using electro-spray mass spectroscopy (Finnigan mat SSQ710).

30 Several other well known methods can be applied by a person skilled in the art to synthesise the peptides.

The peptides of the invention may be used in a method for
35 the treatment of Alzheimer's disease and Down syndrome patients with cells producing frameshift mutant β APP and Ubi-B proteins, which treatment comprises administering at

least one peptide of the present invention *in vivo* or *ex vivo* to a patient in need of such treatment.

In another embodiment the peptides of the invention may be
5 used to vaccinate a human being disposed for Alzheimer's disease, by administering at least one peptide of the present invention to said human being.

It is further considered to be an advantage to administer
10 to a human a mixture of the peptides of this invention, whereby each of the peptides of the invention can bind to different types of HLA class I and/or class II molecules of the individual.

15 It is considered that the peptides may be administered together, either simultaneously or separately, with compounds such as cytokines and/or growth factors, i.e. interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte macrophage colony stimulating factor (GM-CSF), or the like
20 in order to strengthen the immune response as known in the art.

The peptides according to the present invention can be used in a vaccine or a therapeutical composition either alone or
25 in combination with other materials, such as for instance standard adjuvants or in the form of a lipopeptide conjugate which as known in the art can induce high-affinity cytotoxic T lymphocytes, (K. Deres, Nature, Vol.342, (nov.1989)).

30 The peptides according to the present invention may be useful to include in either a peptide or recombinant fragment based vaccine.

The peptides according to the present invention can be included in pharmaceutical compositions or in vaccines together with usual additives, diluents, stabilisers or the like as known in the art.

5

According to this invention, a pharmaceutical composition or vaccine may include the peptides alone or in combination with at least one pharmaceutically acceptable carrier or diluent.

10

Further a vaccine or therapeutical composition can comprise a selection of peptides which are fragments of the mutant β APP and Ubi-B proteins associated with Alzheimer's disease and Down syndrome.

15

The vaccine according to this invention may further be administered to the population in general for example as a mixture of peptides giving rise to T cell immunity against cells in which Alzheimer's disease and Down syndrome connected β APP and Ubi-B frameshift mutations may occur.

20

The peptides according to this invention may be administered as single peptides or as a mixture of peptides. Alternatively the peptides may be covalently linked with each other to form larger polypeptides or even cyclic polypeptides.

25
30

A therapy for Alzheimer's disease and Down syndrome according to the present invention may be administered both *in vivo* or *ex vivo* having as the main goal to elicit specific T cell immunity against the mutant β APP and Ubi-B gene products associated with Alzheimer's disease and Down syndrome.

Further, the frameshift mutant peptides of this invention may be administered to a patient by various routes including but not limited to subcutaneous, intramuscular, intradermal, intraperitoneal, intravenous or the like. In
5 one embodiment the peptides of this invention are administered intradermally. The peptides may be administered at single or multiple injection sites to a patient in a therapeutically or prophylactically effective amount.

10

The peptides of this invention may be administered only once or alternatively several times, for instance once a week over a period of 1-2 months with a repeated sequence later, all according to the need of the patient being
15 treated.

The peptides of this invention can be administered in an amount in the range of 1 microgram (1 µg) to 1 gram (1g) to an average human patient or individual to be vaccinated. It
20 is preferred to use a smaller dose in the range of 1 microgram (1 µg) to 1 milligram (1 mg) for each administration.

25 The invention further encompasses DNA sequences which encodes a frameshift mutation peptide.

The peptides according to the invention may be administered to an individual in the form of DNA vaccines. The DNA encoding these peptides may be in the form of cloned
30 plasmid DNA or synthetic oligonucleotide. The DNA may be delivered together with cytokines, such as IL-2, and/or other co-stimulatory molecules. The cytokines and/or co-stimulatory molecules may themselves be delivered in the form of plasmid or oligonucleotide DNA. The response to a
35 DNA vaccine has been shown to be increased by the presence

of immunostimulatory DNA sequences (ISS). These can take the form of hexameric motifs containing methylated CpG, according to the formula :

5'-purine-purine-CG-pyrimidine-pyrimidine-3'. Our DNA

vaccines may therefore incorporate these or other ISS, in the DNA encoding the peptides, in the DNA encoding the cytokine or other co-stimulatory molecules, or in both. A review of the advantages of DNA vaccination is provided by Tighe et al (1998, *Immunology Today*, 19(2), 89-97).

10

In one embodiment, the DNA sequence encoding the mutant β APP and mutant Ubi-B peptides comprises:

Normal β APP gene sequence (exons 9 and 10).

15

repeat 1

GAG AGG CTT GAG GCC AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG

repeat 2

AGA GAA TGG GAA GAG GCA GAA CGT CAA GCA AAG AAC TTG CCT AAA

20

Mutant β APP gene sequence, GA deleted from repeat 1.

GAG AGG CTT GAG GCC AAG CAC CGA GAG AAT GTC CCA GGT CAT GAG

AGA ATG GGA AGA GGC AGA ACG TCA AGC AAA GAA CTT GCC TAA

Mutant β APP gene sequence, GA deleted from repeat 2.

25

GAG AGG CTT GAG GCC AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG

AGA ATG GGA AGA GGC AGA ACG TCA AGC AAA GAA CTT GCC TAA

Normal Ubi-B gene (exon) sequence.

deletion motif

30

CAC CTG GTC CTG CGT CTG AGA GGT GGT ATG CAG ATC TTC GTG AAG

ACC CTG ACC GGC AAG ACC ATC ACC CTG GAA GTG GAG CCC AGT GAC

Mutant Ubi-B gene sequence, GT deleted from the deletion motif.

CAC CTG GTC CTG CGT CTG AGA GGG TAT GCA GAT CTT CGT GAA GAC
CCT GAC CGG CAA GAC CAT CAC CCT GGA AGT GGA GCC CAG TGA

5

Normal Ubi-B gene (exon 2) sequence.

CAC CTG GTC CTG CGT CTG AGA GGT GGT ATG CAG ATC TTC GTG AAG
CT repeat
ACC CTG ACC GGC AAG ACC ATC ACT CTG GAG GTG GAG CCC AGT GAC

10

Mutant Ubi-B gene sequence, CT deleted from the CT repeat.

CAC CTG GTC CTG CGT CTG AGA GGT GGT ATG CAG ATC TTC GTG AAG
ACC CTG ACC GGC AAG ACC ATC ACT GGA GGT GGA GCC CAG TGA

15

The invention further encompasses vectors and plasmids comprising a DNA sequence encoding at least one frameshift mutant β APP and/or Ubi-B peptide. The vectors include, but are not limited to *E.Coli* plasmid, a Listeria vector and recombinant viral vectors. Recombinant viral vectors include, but are not limited to orthopox virus, canary virus, capripox virus, suipox virus, vaccinia, baculovirus, human adenovirus, SV40, bovine papilloma virus and the like comprising the DNA sequence encoding a mutant β APP and/or Ubi-B peptide.

It is considered that a treatment for Alzheimer's disease and Down syndrome, or prophylaxis for Alzheimer's disease, may be achieved also through the administration of an effective amount of a recombinant virus vector or plasmid comprising at least one insertion site containing a DNA sequence encoding a frameshift mutant peptide to a patient,

whereby the patient's antigen presenting cells are turned into host cells for the vector/plasmid and presentation of HLA/frameshift mutant peptide complex is achieved.

5 A person skilled in the art will find other possible use combinations with the peptides of this invention, and these are meant to be encompassed by the present claims.

The peptides according to this invention may be produced by conventional processes as known in the art, such as chemical peptide synthesis, recombinant DNA technology or protease cleavage of a protein or peptide encoded by frameshift mutated β APP gene and Ubi-B gene. One method for chemical synthesis is elucidated in the description 15 below.

Through the present invention the following advantages are achieved:

20 It offers a possibility to treat patients suffering from Alzheimer's disease and Down syndrome connected with frameshift mutant β APP and Ubi-B gene products, who known at present do not have any good treatment alternatives.

25 Furthermore it offers a possibility to vaccinate humans prophylactically against the onset of Alzheimer's disease.

Claims

1. A peptide characterised in that it

5 a) is at least 8 amino acids long and is a fragment of a mutant β APP and/or Ubi-B protein arising from a frameshift mutation associated with Alzheimer's disease and/or Down syndrome;

10 and

b) consists of at least one amino acid of the mutant part of the mutant β APP and/or Ubi-B protein;

15 and

c) comprises 0-10 amino acids corresponding to the carboxyl terminus of the normal part of the protein sequence preceding the amino terminus of the mutant sequence and may 20 further extend to the carboxyl terminus of the mutant part of the protein as determined by a new stop codon generated by the relevant frameshift mutation;

and

25

d) induces, either in its full length or after processing by antigen presenting cells, T cell responses.

2. A peptide according to claim 1 characterised in that it 30 contain 8-25 amino acids.

3. A peptide according to claim 1 characterised in that it contain 9-20 amino acids.

4. A peptide according to claim 1 characterised in that it contain 9-16 amino acids.

5. A peptide according to claim 1 characterised in that it contain 8-12 amino acids.

6. A peptide according to claim 1 characterised in that it contain 20-25 amino acids.

10 7. A peptide according to claim 1 characterised in that it contains 9 amino acids.

8. A peptide according to claim 1 characterised in that it contains 12 amino acids.

15 9. A peptide according to claim 1 characterised in that it contains 13 amino acids.

20 10. A peptide according to claim 1 characterised in that it is selected from a group of peptides having the following sequence identity numbers:

seq id no. 1 - seq id no. 10 or a fragment of any of these.

25 11. A pharmaceutical composition comprising a peptide according to any of the above claims and a pharmaceutically acceptable carrier or diluent.

30 12. A vaccine for Alzheimer's disease comprising a peptide according to any of the claims 1-10 and a pharmaceutically acceptable carrier or diluent.

35 13. Use of a peptide according to any of the claims 1-10 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

14. Method for vaccination of a person disposed for or afflicted with Alzheimer's disease, consisting of administering at least one peptide according to the claims 1-10, one or more times, in an amount sufficient for

5 induction of specific T-cell immunity to mutant β APP and/or mutant Ubi-B peptides associated with Alzheimer's disease and/or Down syndrome.

15. Method according to claim 14 wherein the amount of the

10 peptides is in the range of 1 microgram (1 μ g) to 1 gram (1g) and preferentially in the rage of 1 microgram (1 μ g) to 1 milligram (1 mg) for each administration.

16. Method for treatment of a patient afflicted with

15 Alzheimer's disease or Down syndrome, by stimulating *in vivo* or *ex vivo* with peptides according to the claims 1-10.

17. Method according to claim 16 wherein the amount of the

peptides used is in the range of 1 microgram (1 μ g) to 1

20 gram (1g) and preferentially in the rage of 1 microgram (1 μ g) to 1 milligram (1 mg) for each administration.

18. An isolated DNA sequence comprising a DNA sequence or

variants thereof encoding a frameshift mutant peptide

25 according to claim 1.

19. An isolated DNA sequence according to claim 18 encoding

peptides comprising seq. id. no: 1-10 or variants thereof.

30 20. Use of a DNA sequence according to any of the claims

18-19 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

21. Method for treatment of a person disposed for or afflicted with Alzheimer's disease or afflicted with Down syndrome, by stimulating *in vivo* or *ex vivo* with DNA sequences according to the claims 18-19.

5

22. A plasmid or virus vector comprising DNA sequences of claim 18 encoding a frameshift mutant β APP peptide and/or Ubi-B peptide associated with Alzheimer's disease or Down syndrome.

10

23. A vector according to claim 22 wherein the vector is *E.Coli* plasmid, a *Listeria* vector and recombinant viral vectors. Recombinant viral vectors include, but are not limited to orthopox virus, canary virus, capripox virus, suipox virus, vaccinia, baculovirus, human adenovirus, SV40 or bovine papilloma virus.

15

24. Use of a plasmid or virus vector according to claim 22 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

20

25. Method for treatment of a person disposed for or afflicted with Alzheimer's disease or afflicted with Down syndrome, by stimulating *in vivo* or *ex vivo* with plasmids or virus vectors according to claim 22.

25

SEQUENCE LISTINGCOMMON FOR ALL SEQUENCES.

SEQUENCE TYPE: Peptide

SEQUENCE UNIT: Amino Acid

TOPOLOGY: Linear

SEQUENCE ID NO: 1

SEQUENCE LENGTH: 19 amino acids

N V P G H E R M G R G R T S S K E L A
1 5 10 15**SEQUENCE ID NO: 2**

SEQUENCE LENGTH: 27 amino acids

R L E A K H R E N V P G H E R M G R G R T S S K E L A
1 5 10 15 20 25**SEQUENCE ID NO: 3**

SEQUENCE LENGTH: 17 amino acids

R L E A K H R E N V P G H E R M G
1 5 10 15**SEQUENCE ID NO: 4**

SEQUENCE LENGTH: 12 amino acids

M G R G R T S S K E L A
1 5 10**SEQUENCE ID NO: 5**

SEQUENCE LENGTH: 15 amino acids

E R M S Q V M R M G R G R T S
1 5 10 15

SEQUENCE ID NO: 6

SEQUENCE LENGTH: 20 amino acids

Y A D L R E D P D R Q D H H P G S G A Q
1 5 10 15 20

SEQUENCE ID NO: 7

SEQUENCE LENGTH: 28 amino acids

SEQUENCE ID NO: 8

SEQUENCE LENGTH: 17 amino acids

H L V L R L R G Y A D L R E D P D
1 5 10 15

SEQUENCE ID NO: 9

SEQUENCE LENGTH: 5 amino acids

G G G A Q
1 5

SEQUENCE ID NO: 10

SEQUENCE LENGTH: 13 amino acids

T L T G K T I T G G G A Q
1 5 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00141

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/435, A61K 39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9712992 A2 (ROYAL NETHERLANDS ACADEMY OF ARTS AND SCIENCES ET AL), 10 April 1997 (10.04.97), table 7 --	1-25
X	SCIENCE, Volume 279, January 1998, Fred W. van Leeuwen et al, "Frameshift Mutants of beta Amyloid Precursor Protein and Ubiquitin-B in Alzheimer's and Down Patients" page 242 - page 247 --	1-26
X	WO 9532731 A2 (THE CHANCELLOR MASTERS AND SCHOLARS OF THE UNIVERSITY OF OXFORD ET AL), 7 December 1995 (07.12.95), page 3, line 8 - page 4, line 26 --	11-25

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 October 1999

Date of mailing of the international search report

22-10-1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00141

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9845322 A2 (ROYAL NETHERLANDS ACADEMY OF ARTS AND SCIENCES ET AL), 15 October 1998 (15.10.98), claim 24 and the whole document -- -----	1-25

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/NO99/00141**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: **14-17, 21 and 25**
because they relate to subject matter not required to be searched by this Authority, namely:
See extra sheet

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO99/00141

Claims 14-17, 21 and 25 relates to methods of treatment of the human or animal body by therapy practised on the human or animal body/ Rule 39.1. (iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/09/99

International application No.

PCT/NO 99/00141

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9712992 A2	10/04/97	AU 7142796 A GB 9520080 D		28/04/97 00/00/00
WO 9532731 A2	07/12/95	AU 2623795 A EP 0762891 A GB 9410922 D JP 10504702 T		21/12/95 19/03/97 00/00/00 12/05/98
WO 9845322 A2	15/10/98	AU 7071598 A		30/10/98



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/00		A2	(11) International Publication Number: WO 99/60021															
			(43) International Publication Date: 25 November 1999 (25.11.99)															
<p>(21) International Application Number: PCT/US99/10953</p> <p>(22) International Filing Date: 19 May 1999 (19.05.99)</p> <p>(30) Priority Data:</p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">124550</td> <td style="width: 33%;">19 May 1998 (19.05.98)</td> <td style="width: 33%;">IL</td> </tr> <tr> <td>PCT/US98/14715</td> <td>21 July 1998 (21.07.98)</td> <td>US</td> </tr> <tr> <td>09/218,277</td> <td>22 December 1998 (22.12.98)</td> <td>US</td> </tr> </table> <p>(71) Applicant: YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; P.O. Box 95, 76100 Rehovot (IL).</p> <p>(71) Applicant (for SD only): MCINNIS, Patricia, A. [US/US]; Apartment #203, 2325 42nd Street N.W., Washington, DC 20007 (US).</p> <p>(72) Inventors: EISENBACH-SCHWARTZ, Michal; Rupin Street 5, 76353 Rehovot (IL). COHEN, Irun, R.; Hankin Street 11, 76343 Rehovot (IL). BESERMAN, Pierre; 76834 Moshav Sitriya (IL). MOSONEGO, Alon; Ben-Yosef, 73112 Kfar Hanoar Ben-Shemen (IL). MOALEM, Gila; Bosel Street 27, 76405 Rehovot (IL).</p> <p>(74) Agent: BROWDY, Roger, L.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street N.W., Washington, DC 20004 (US).</p>		124550	19 May 1998 (19.05.98)	IL	PCT/US98/14715	21 July 1998 (21.07.98)	US	09/218,277	22 December 1998 (22.12.98)	US	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>							
124550	19 May 1998 (19.05.98)	IL																
PCT/US98/14715	21 July 1998 (21.07.98)	US																
09/218,277	22 December 1998 (22.12.98)	US																
<p>(54) Title: ACTIVATED T CELLS, NERVOUS SYSTEM-SPECIFIC ANTIGENS AND THEIR USES</p> <p>(57) Abstract</p> <p>Compositions and methods are provided for treating injury to or disease of the central or peripheral nervous system. In one embodiment, treatment is effected using activated T cells that recognize an antigen of the nervous system or a peptide derived therefrom or a derivative thereof to promote nerve regeneration or to prevent or inhibit neuronal degeneration within the nervous system. Treatment involves administering an NS-specific antigen or peptide derived therefrom or a derivative thereof, or a nucleotide sequence encoding said antigen or peptide, to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the nervous system, either the central nervous system or the peripheral nervous system. The NS-specific activated T cells can be administered alone or in combination with NS-specific antigen or peptide derived therefrom or a derivative thereof or a nucleotide sequence encoding said antigen or peptide, or any combination thereof.</p>																		
<p>The bar chart displays the number of T cells (y-axis, 0 to 300) for various treatments (x-axis) in both injured and uninjured optic nerves. The treatments are grouped by nerve type: Injured optic nerve (T_Hor, T_p27, T_m, PBS) and Uninjured optic nerve (T_Hor, T_p27, T_m, PBS). Error bars are shown for each bar.</p> <table border="1"> <thead> <tr> <th>Treatment</th> <th>Injured optic nerve (approx.)</th> <th>Uninjured optic nerve (approx.)</th> </tr> </thead> <tbody> <tr> <td>T_{Hor}</td> <td>250</td> <td>50</td> </tr> <tr> <td>T_{p27}</td> <td>170</td> <td>50</td> </tr> <tr> <td>T_m</td> <td>160</td> <td>50</td> </tr> <tr> <td>PBS</td> <td>50</td> <td>50</td> </tr> </tbody> </table>				Treatment	Injured optic nerve (approx.)	Uninjured optic nerve (approx.)	T _{Hor}	250	50	T _{p27}	170	50	T _m	160	50	PBS	50	50
Treatment	Injured optic nerve (approx.)	Uninjured optic nerve (approx.)																
T _{Hor}	250	50																
T _{p27}	170	50																
T _m	160	50																
PBS	50	50																

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**ACTIVATED T CELLS, NERVOUS SYSTEM-SPECIFIC ANTIGENS
AND THEIR USES**

Field of the Invention

The present invention relates to compositions and methods for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury or disease of the nervous system (NS). In certain embodiments, activated antiself T cells, an NS-specific antigen or peptide derived therefrom or a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used to promote nerve regeneration or to prevent or inhibit neuronal degeneration caused by injury or disease of nerves within the central nervous system or peripheral nervous system of a human subject. The compositions of the present invention may be administered alone or may be optionally administered in any desired combination.

Background of the Invention

The nervous system comprises the central (CNS) and the peripheral (PNS) nervous system. The central nervous system is composed of the brain and spinal cord; the peripheral nervous system consists of all of the other neural elements, namely the nerves and ganglia outside of the brain and spinal cord.

Damage to the nervous system may result from a traumatic injury, such as penetrating trauma or blunt trauma, or a disease or disorder, including but not limited to Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS), diabetic neuropathy, senile dementia, and ischemia.

Maintenance of central nervous system integrity is a complex "balancing act" in which compromises are struck with the immune system. In most tissues, the immune system plays an essential part in protection, repair, and healing. In the central nervous system, because of its unique immune privilege, immunological reactions are relatively limited (Streilein, J.W., 1993, *Curr. Opin. Immunol.* 5:428-423; Streilein, J.W., *Science* 270:1158-1159). A growing body of evidence indicates

that the failure of the mammalian central nervous system to achieve functional recovery after injury is a reflection of an ineffective dialog between the damaged tissue and the immune system. For example, the restricted communication between the central nervous system and blood-borne macrophages affects the capacity of axotomized axons to regrow; transplants of activated macrophages can promote central nervous system regrowth (Lazarov Spiegler, O., et al, 1996, FASEB J. 19:1296-1302; Rapalino, O. et al., 1998, Nature Med. 4:814-821).

Activated T cells have been shown to enter the central nervous system parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a central nervous system antigen seem to persist there (Hickey, W.F. et al., 1991, J. Neurosci. Res. 28:254-260; Werkele, H., 1993, In The Blood-Brain Barrier, Pardridge, Ed., Raven Press, Ltd. New York, 67-85; Kramer, R. et al., 1995, Nature Med. 1(11):1162-1166)). T cells reactive to antigens of central nervous system white matter, such as myelin basic protein (MBP), can induce the paralytic disease experimental autoimmune encephalomyelitis (EAE) within several days of their inoculation into naive recipient rats (Ben Nun, A., et al., 1981, Eur. J. Immunol. 11:195-199). Anti-MBP T cells may also be involved in the human disease multiple sclerosis (Ota, K. et al., 1990 Nature 346:183-187; Martin, R. 1997, J. Neural Transm. Suppl. 49:53-67). However, despite their pathogenic potential, anti-MBP T cell clones are present in the immune systems of healthy subjects (Burns, J., et al. 1983, Cell Immunol. 81:435-440; Pette, M. et al., 1990, Proc. Natl. Acad. Sci. USA 87:7968-7972; Martin, R. et al., 1990, J. Immunol. 145:540-548; Schiuesener, H.J., et al., 1985, J. Immunol. 135:3128-3133). Activated T cells, which normally patrol the intact central nervous system, transiently accumulate at sites of central nervous system white matter lesions (Hirschberg, D.L., et al., 1998, J. Neuroimmunol. 89:88-96).

A catastrophic consequence of central nervous system injury is that the primary damage is often compounded by the gradual secondary loss of adjacent neurons that apparently were undamaged, or only marginally damaged, by the initial injury

(Faden, A. I., et al., 1992, Trends Pharmacol. Sci. 13:29-35; Faden, A.I., 1993, Crit. Rev. Neurobiol. 7:175-186; McIntosh, T.K., 1993, J. Neurotrauma 10:215-261). The primary lesion causes changes in extracellular ion concentrations, elevation of amounts of free radicals, release of neurotransmitters, depletion of growth factors, and local inflammation. These changes trigger a cascade of destructive events in the adjacent neurons that initially escaped the primary injury (Lynch, D.R. et al., 1994, Curr. Opin. Neurol. 7:510-516; Bazan, N.G. et al., 1995, J. Neurotrauma 12:791-814; Wu, D. et al., 1994, J. Neurochem. 62:37-44). This secondary damage is mediated by activation of voltage-dependent or agonist-gated channels, ion leaks, activation of calcium-dependent enzymes such as proteases, lipases and nucleases, mitochondrial dysfunction and energy depletion, culminating in neuronal cell death (Yoshina, A. et al., 1991 Brain Res. 561:106-119; Hovda, D.A. et al., 1991, Brain Res. 567:1-10; Zivin, J.A., et al, 1991 Sci. Am. 265:56-63; Yoles, E. et al., 1992, Invest. Ophthalmol. Vis. Sci. 33:3586-3591). The widespread loss of neurons beyond the loss caused directly by the primary injury has been called "secondary degeneration."

Another tragic consequence of central nervous system injury is that neurons in the mammalian central nervous system do not undergo spontaneous regeneration following an injury. Thus, a central nervous system injury causes permanent impairment of motor and sensory functions.

Spinal cord lesions, regardless of the severity of the injury, initially result in a complete functional paralysis known as spinal shock. Some spontaneous recovery from spinal shock may be observed, starting a few days after the injury and tapering off within three to four weeks. The less severe the insult, the better the functional outcome. The extent of recovery is a function of the amount of undamaged tissue minus the loss due to secondary degeneration. Recovery from injury would be improved by neuroprotective treatment that could reduce secondary degeneration.

Citation or identification of any reference in this section or any other part of this application shall not be

construed as an admission that such reference is available as prior art to the invention.

SUMMARY OF THE INVENTION

The present invention is directed to methods and compositions for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury to or disease of the nervous system (NS). The present invention is based in part on the applicants' unexpected discovery that activated T cells that recognize an antigen of the NS of the patient promote nerve regeneration or confer neuroprotection. As used herein, "neuroprotection" refers to the prevention or inhibition of degenerative effects of injury or disease in the NS. Until recently, it was thought that the immune system excluded immune cells from participating in nervous system repair. It was quite surprising to discover that NS-specific activated T cells can be used to promote nerve regeneration or to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or PNS.

"Activated T cell" as used herein includes (i) T cells that have been activated by exposure to a cognate antigen or peptide derived therefrom or derivative thereof and (ii) progeny of such activated T cells. As used herein, a cognate antigen is an antigen that is specifically recognized by the T cell antigen receptor of a T cell that has been previously exposed to the antigen. Alternatively, the T cell which has been previously exposed to the antigen may be activated by a mitogen, such as phytohemagglutinin (PHA) or concanavalin A.

In one embodiment, the present invention provides pharmaceutical compositions comprising a therapeutically effective amount of NS-specific activated T cells and methods for using such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in an amount which is effective to ameliorate the effects of an injury or disease of the NS. "NS-specific activated T cell" as used herein refers to an activated T cell having specificity for an antigen of the NS of a patient. The antigen used to confer the specificity to the T cells may be a self NS-antigen

of the patient, a peptide derived therefrom, or an NS-antigen of another individual or even another species, or a peptide derived therefrom, as long as the activated T cell recognizes an antigen in the NS of the patient.

The NS-specific activated T cells are used to promote nerve regeneration or to prevent or inhibit the effects of disease. If the disease being treated is an autoimmune disease, in which the autoimmune antigen is an NS antigen, the T cells which are used in accordance with the present invention for the treatment of neural damage or degeneration caused by such disease are preferably not activated against the same autoimmune antigen involved in the disease. While the prior art has described methods of treating autoimmune diseases by administering activated T cells to create a tolerance to the autoimmune antigen, the T cells of the present invention are not administered in such a way as to create tolerance, but are administered in such a way as to create accumulation of the T cells at the site of injury or disease so as to facilitate neural regeneration or to inhibit neural degeneration.

The prior art also discloses uses of immunotherapy against tumors, including brain tumors, by administering T cells specific to an NS antigen in the tumor so that such T cells may induce an immune system attack against the tumors. The present invention is not intended to comprehend such prior art techniques. However, the present invention is intended to comprehend the inhibition of neural degeneration or the enhancement of neural regeneration in patients with brain tumors by means other than the prior art immunotherapy of brain tumors. Thus, for example, NS-specific activated T cells, which are activated to an NS antigen of the patient other than an antigen which is involved in the tumor, would be expected to be useful for the purpose of the present invention and would not have been suggested by known immunotherapy techniques.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in which the amount is

effective to activate T cells *in vivo* or *in vitro*, wherein the activated T cells inhibit or ameliorate the effects of an injury or disease of the NS. "NS-specific antigen" as used herein refers to an antigen that specifically activates T cells such that following activation the activated T cells accumulate at a site of injury or disease in the NS of the patient. In one embodiment, the peptide derived from an NS-specific antigen is a "cryptic epitope" of the antigen. A cryptic epitope activates specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen. In another embodiment, the peptide derived from an NS-specific antigen is an immunogenic epitope of the antigen. "Derivatives" of NS-specific antigens or peptides derived therefrom as used herein refers to analogs or chemical derivatives of such antigens or peptides as described below, see Section 5.2.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or for preventing or inhibiting neuronal degeneration in the CNS or PNS in which the amount is effective to ameliorate the effects of an injury or disease of the NS.

In the practice of the invention, therapy for amelioration of effects of injury or disease comprising administration of NS-specific activated T cells may optionally be in combination with an NS-specific antigen or peptide derived therefrom.

Additionally, oral administration of NS-specific antigen or a peptide derived therefrom, can be combined with active immunization to build up a critical T cell response immediately after injury.

In another embodiment cell, banks can be established to store NS sensitized T cells for neuroprotective treatment of individuals at a later time, as needed. In this case, autologous T cells may be obtained from an individual. Alternatively, allogeneic or semi-allogeneic T cells may be stored such that a bank of T cells of each of the most common

MHC-class II types are present. In case an individual is to be treated for an injury, preferably autologous stored T cells are used, but, if autologous T cells are not available, then cells should be used which share an MHC type II molecule with the patient, and these would be expected to be operable in that individual. The cells are preferably stored in an activated state after exposure to an NS antigen or peptide derived therefrom. However, the cells may also be stored in a resting state and activated once they are thawed and prepared for use. The cell lines of the bank are preferably cryopreserved. The cell lines are prepared in any way which is well known in the art. Once the cells are thawed, they are preferably cultured prior to injection in order to eliminate non-viable cells. During this culturing, the cells can be activated or reactivated using the same NS antigen or peptide as used in the original activation. Alternatively, activation may be achieved by culturing in the presence of a mitogen, such as phytohemagglutinin (PHA) or concanavalin A (preferably the former). This will place the cells into an even higher state of activation. The few days that it takes to culture the cells should not be detrimental to the patient as the treatment in accordance with the present invention may occur any time up to a week or more after the injury in order to still be effective. Alternatively, if time is of the essence, the stored cells may be administered immediately after thawing.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a bar graph showing the presence of T cells in uninjured optic nerve or in injured optic nerve one week after injury. Adult Lewis rats were injected with activated T cells of the anti-MBP (T_{MBP}), anti-OVA (T_{OVA}), anti-p277 (T_{p277}) lines, or with PBS, immediately after unilateral crush injury of the optic nerve. Seven days later, both the injured and uninjured optic nerves were removed, cryosectioned and analyzed immunohistochemically for the presence of immunolabeled T cells. T cells were counted at the site of injury and at randomly selected areas in the uninjured optic nerves. The histogram shows the mean number of T cells per $\text{mm}^2 \pm \text{s.e.m.}$, counted in two to three sections of each nerve. Each

group contained three to four rats. The number of T cells was considerably higher in injured nerves of rats injected with anti-MBP, anti-OVA or anti-p277 T cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells and the T cell numbers in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

Fig. 2 is a bar graph illustrating that T cells specific to MBP, but not of OVA or p277 or hsp60, protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP, anti-OVA or anti-p277 T cells, or with PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after the primary injury (42% of neurons remained undamaged after the primary injury). The neuroprotective effect of anti-MBP T cells compared with that of PBS was significant ($P<0.001$, one-way ANOVA). Anti-OVA T cells or anti-p277 T cells did not differ significantly from PBS in their effects on the protection of neurons that had escaped primary injury ($P>0.05$, one-way ANOVA). The results are a summary of five experiments. Each group contained five to ten rats.

Figs. 3 (A-C) present photomicrographs of retrogradely labeled retinas of injured optic nerves of rats. Immediately after unilateral crush injury of their optic nerves, rats were injected with PBS (Fig. 3A) or with activated anti-p277 T cells (Fig. 3B) or activated anti-MBP T cells (Fig. 3C). Two weeks later, the neurotracer dye 4-Di-10-Asp was

applied to the optic nerves, distal to the site of injury. After 5 days, the retinas were excised and flat-mounted. Labeled (surviving) RCGs, located at approximately the same distance from the optic disk in each retina, were photographed.

Figs. 4(A-B) are graphs showing that clinical severity of EAE is not influenced by an optic nerve crush injury. For the results presented in Fig. 4A, Lewis rats, either uninjured (dash line) or immediately after optic nerve crush injury (solid line), were injected with activated anti-MBP T cells. EAE was evaluated according to a neurological paralysis scale. [Data points represent \pm s.e.m.] These results represent a summary of three experiments. Each group contained five to nine rats. Fig. 4B shows that the number of RGCs in the uninjured optic nerve is not influenced by injection of anti-MBP T cells. Two weeks after the injection of anti-MBP T cells or PBS, 4-Di-10Asp was applied to the optic nerves. After 5 days the retinas were excised and flat-mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk) in each retina were counted and the average number per mm^2 was calculated. There was no difference between the numbers of labeled RGCs in rats injected with anti-MBP T cells (T_{MBP}) and in PBS-injected control rats.

Fig. 5 is a bar graph showing that T cells specific to p51-70 of MBP protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP T cells, anti-p51-70 T cells, or PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after primary injury. Compared with

that of PBS treatment, the neuroprotective effects of anti-MBP anti-p51-70 T cells were significant ($P<0.001$, one-way ANOVA).

Figs. 6(A-B) are graphs showing that anti-MBP T cells increase the compound action potential (CAP) amplitudes of injured optic nerves. Immediately after optic nerve injury, rats were injected with either PBS or activated anti-MBP T cells (T_{MBP}). Two weeks later, the CAPs of injured (Fig. 6A) and uninjured (Fig. 6B) nerves were recorded. There were no significant differences in mean CAP amplitudes between uninjured nerves obtained from PBS-injected and T cell-injected rats ($n=8$; $p=0.8$, Student's t-test). The neuroprotective effect of anti-MBP T cells (relative to PBS) on the injured nerve on day 14 after injury was significant ($n=8$, $p=0.009$, Student's t-test).

Figs. 7(A-B) are graphs showing recovery of voluntary motor activity as a function of time after contusion, with and without injection of autoimmune anti-MBP T cells. (7A) Twelve rats were deeply anesthetized and laminectomized, and then subjected to a contusion insult produced by a 10 gram weight dropped from a height of 50 mm. Six of the rats, selected at random, were then inoculated i.p. with 10^7 anti-MBP T cells and the other six were inoculated with PBS. At the indicated time points, locomotor behavior in an open field was scored by observers blinded to the treatment received by the rats. Results are expressed as the mean values for each group. The vertical bars indicate SEM. Differences tested by repeated ANOVA, including all time points, were significant ($p<0.05$). (7B) A similar experiment using five PBS-treated animals and six animals treated with anti-MBP T cells were all subjected to a more severe contusion. At the indicated time points, locomotor behavior in an open field was scored. The results are expressed as the mean values for each group. The vertical bars indicate S.E.M. Rats in the treated group are represented by open circles and rats in the control group are represented by black circles. Horizontal bars show the median values. The inset shows the median plateau values of the two groups.

Figs 8(A-C) show retrograde labeling of cell bodies at the red nucleus in rats treated with autoimmune anti-MBP T cells (8A) and in control injured (8B) rats. Three months

after contusion and treatment with anti-MBP T cells, some rats from both the treated and the control groups were re-anesthetized and a dye was applied below the site of the contusion. After five to seven days the rats were again deeply anesthetized and their brains were excised, processed, and cryosectioned. Sections taken through the red nucleus were inspected and analyzed qualitatively and quantitatively under fluorescent and confocal microscopes. Significantly, more labelled nuclei were seen in the red nuclei of rats treated with anti-MBP T cells (8A) than in the red nuclei of PBS-treated rats (8B). The quantitative differences are shown in the bar graph (8C) and were obtained from animals with scores of 10 and 11 in the T cell treated group and scores of 6 in the control group. The bar graph shows mean \pm SD.

Fig. 9 is a series of photographs showing diffusion-weighted imaging of contused spinal cord treated with anti-MBP T cells. Spinal cords of MBP-T cell-treated and PBS-treated animals (with locomotion scores of 10 and 8, respectively) were excised under deep anesthesia, immediately fixed in 4% paraformaldehyde solution, and placed into 5 mm NMR tubes. Diffusion anisotropy was measured in a Bruker DMX 400 widebore spectrometer using a microscopy probe with a 5-mm Helmholtz coil and actively shielded magnetic field gradients. A multislice pulsed gradient spin echo experiment was performed with 9 axial slices, with the central slice positioned at the center of the spinal injury. Images were acquired with TE of 31 ms, TR of 2000 ms, a diffusion time of 15 ms, a diffusion gradient duration of 3 ms, field of view 0.6 mm, matrix size 128 x 128, slice thickness 0.5 mm, and slice separation of 1.18 mm. Four diffusion gradient values of 0, 28, 49, and 71 g/cm were applied along the read direction (transverse diffusion) or along the slice direction (longitudinal diffusion). Diffusion anisotropy is manifested by increased signal intensity in the images with the highest transverse diffusion gradient relative to the longitudinal diffusion gradient. The excised spinal cords of a PBS-treated rat and in the rat treated with MBP-T cells were subjected to diffusion-weighted MRI analysis. In the PBS-treated injured control, diffusion anisotropy was seen mainly in sections near the proximal and distal stumps of the

cord, with low anisotropy in sections taken through the site of injury. In contrast, in the treated rat, higher levels of diffusion anisotropy can be seen in sections taken through the site of injury.

Fig. 10 is a graph illustrating inhibition of secondary degeneration after optic nerve crush injury in adult rats. See text, Section 8, for experimental details. Rats were injected intradermally through the footpads with a 21-mer peptide based on amino acid residues 35-55 (MOG p35-55) of myelin/oligodendrocyte glycoprotein (chemically synthesized at the Weizmann Institute, Israel) (50 μ /animal) or PBS ten days prior to optic nerve crush injury or MOG p35-55 in the absence of crush injury. MOG p35-55 was administered with Incomplete Freund's Adjuvant. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in rats injected with PBS or MOG p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

Fig. 11 is a graph illustrating inhibition in adult rats of secondary degeneration after optic nerve crush injury by MBP. See text, Section 9, for experimental details. MBP (Sigma, Israel) (1 mg in 0.5 ml saline) was administered orally to adult rats by gavage using a blunt needle. MBP was administered 5 times, i.e., every third day beginning two weeks prior to optic nerve crush injury. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in treated rats was expressed as a percentage of the total number of neurons in untreated rats following the injury.

Figs. 12 (A-F) show expression of B7 costimulatory molecules in intact and injured rat optic nerve. Optic nerves were excised from adult Lewis rats before (12A, 12B) and three days after injury (12C, 12D, 12E) and analyzed immunohistochemically for expression of the B7 costimulatory molecule. The site of injury was delineated by GFAP staining. Using calibrated cross-action forceps, the right optic nerve was subjected to a mild crush injury 1-2 mm from the eye. The uninjured contralateral nerve was left undisturbed. Immunohistochemical analysis of optic nerve antigens was

performed as follows. Briefly, longitudinal cryosections of the excised nerves (20 μm thick) were picked up onto gelatin-coated glass and fixed with ethanol for ten minutes at room temperature. The sections were washed and incubated for one hour at room temperature with mouse monoclonal antibody to rat GFAP (BioMakor, Israel), diluted 1:100, and with antibodies to B7.2 costimulatory molecule and the B7.1 costimulatory molecule (PHARMINGEN, San Diego, CA), diluted 1:25. The sections were washed again and incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum protein) (Jackson ImmunoResearch, West Grove, PA), for one hour at room temperature. All washing solutions contained PBS and 0.05% Tween-20. All diluting solutions contained PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were treated with glycerol containing 1,4-diazobicyclo-(2,2,2)-octane and were then viewed with a Zeiss microscope. Note the morphological changes of the B7.2 positive cells after injury, from a rounded (12A, 12B) to a star-like shape (12C, 12D). The B7.2 positive cells were present at a higher density closer to the injury site (12E). Expression of B7.1 was detectable only from day seven and only at the injured site (12F).

Figs. 13 A-C show immunohistochemical analysis of T cells, macrophages or microglia, and B7.2 costimulatory molecules in the injured optic nerves of rats fed MBP. Lewis rats aged 6-8 weeks were fed 1 mg of bovine MBP (Sigma, Israel) (2 mg MBP/ml PBS) or 0.5 ml PBS only every other day by gastric intubation using a stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ) (Chen, Y., Kuchroo, V.K., Inobe, J. Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237-1240, 1994). Ten days after starting MBP the right optic nerves were subjected to calibrated crush injury, as described for Figure 12. Three days later the nerves were excised and prepared for immunohistochemical analysis of T cells using mouse monoclonal antibodies to T cell receptor 11, diluted 1:25, macrophages or microglia using anti-ED1 antibodies (Serotek, Oxford, U.K) diluted 1:250, astrocytes using anti-GFAP antibodies and B7.2

costimulatory molecules as described for Fig. 12. There were no significant quantitative differences in T cells or in ED-1 positive cells between injured optic nerves of PBS-fed (13A) and MBP-fed (13B) rats. The number of B7.2 positive cells at the site of injury of MBP-fed rats (13C) should be noted, as compared with injured controls (Fig. 12E).

Fig. 14 is a graph showing the slowing of neuronal degeneration in rats with orally induced tolerance to MBP. Lewis rats were fed 1 mg MBP daily, or every other day, or 4 times a day at two hour intervals for five consecutive days. Control animals were given PBS or the non-self antigen OVA (Sigma, Israel). Ten days after the start of MBP ingestion, the right optic nerves were subjected to a calibrated mild crush injury. Two weeks later the RGCs were retrogradely labelled by application of the fluorescent lipophilic dye, 4-(4-didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the site of injury, as described. Briefly, complete axotomy was performed 1-2 mm from the distal border to the injury site, and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were immediately deposited at the site of the lesion. Retrograde labelling of RGCs by the dye gives a reliable indication of the number of still-functioning neurons, as only intact axons can transport the dye to their cell bodies in the retina. Six days after dye application, the retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labelled ganglion cells by fluorescence microscopy. RGCs were counted from three different regions in the retina. The results are expressed as normalized percentage of each retina to untreated injured animal mean of the same experiment. The median of each group is shown as a bar (Control vs. MBP OTx4 ** P<0.01; Control vs. MBP OT ** P, 0.01; Control vs. OVA OT ns P>0.05.

Fig. 15 shows the nucleotide sequence of rat myelin basic protein gene, SEQ ID NO:1, Genbank accession number M25889 (Schaich et al., Biol. Chem. 367:825-834, 1986).

Fig. 16 shows the nucleotide sequence of human myelin basic protein gene, SEQ ID NO:2, Genbank accession number

M13577 (Kamholz et al., Proc. Natl. Acad. Sci. U.S.A. 83(13): 4962-4966, 1986).

Figs 17 (A-F) show the nucleotide sequences of human myelin proteolipid protein gene exons 1-7, SEQ ID NOs:3-8, respectively, Genbank accession number M15026-M15032 respectively (Diehl et al., Proc. Natl. Acad. Sci. U.S.A. 83(24):9807-9811, 1986; published erratum appears in Proc Natl Acad Sci U.S.A. 86(6):617-8, 1991).

Fig. 18 shows the nucleotide sequence of human myelin oligodendrocyte glycoprotein gene, SEQ ID NO:9, Genbank accession number Z48051 (Roth et al., submitted (17-Jan-1995) Roth, CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

Fig. 19 shows the nucleotide sequence of rat proteolipid protein and variant, SEQ ID NO:10, Genbank accession number M16471 (Nave et al., Proc. Natl. Acad. Sci. U.S.A. 84:600-604, 1987).

Fig. 20 shows the nucleotide sequence of rat myelin-associated glycoprotein, SEQ ID NO:11, Genbank accession number M14871 (Arquint et al., Proc. Natl. Acad. Sci. USA 84:600-604, 1987).

Fig. 21 shows the amino acid sequence of human myelin basic protein, SEQ ID NO:12, Genbank accession number 307160 (Kamholz et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83(13):4962-4966, 1986).

Fig. 22 shows the amino acid sequence of human proteolipid protein, SEQ ID NO:13, Genbank accession number 387028.

Fig. 23 shows the amino acid sequence of human myelin oligodendrocyte glycoprotein, SEQ ID NO:14, Genbank accession number 793839 (Roth et al., Genomics 28(2):241-250, 1995; Roth Submitted (17-JAN-1995) Roth CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

DETAILED DESCRIPTION OF THE INVENTION

Merely for ease of explanation, the detailed description of the present invention is divided into the following subsections: (1) NS-specific activated T cells; (2)

NS-specific antigens, peptides derived therefrom and derivatives thereof; (3) nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; (4) therapeutic uses of non-recombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; and (5) formulations and modes of administration of nonrecombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom.

5.1 NS-SPECIFIC ACTIVATED T CELLS

NS-specific activated T cells (ATCs) can be used for ameliorating or inhibiting the effects of injury or disease of the CNS or PNS that result in NS degeneration or for promoting regeneration in the NS, in particular the CNS.

The NS-specific activated T cells are preferably autologous, most preferably of the CD4 and/or CD8 phenotypes, but they may also be allogeneic T cells from related donors, e.g., siblings, parents, children, or HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

In addition to the use of autologous T cells isolated from the subject, the present invention also comprehends the use of semi-allogeneic T cells for neuroprotection. These T cells may be prepared as short- or long-term lines and stored by conventional cryopreservation methods for thawing and administration, either immediately or after culturing for 1-3 days, to a subject suffering from injury to the central nervous system and in need of T cell neuroprotection.

The use of semi-allogeneic T cells is based on the fact that T cells can recognize a specific antigen epitope presented by foreign antigen presenting cells (APC), provided that the APC express the MHC molecule, class I or class II, to which the specific responding T cell population is restricted, along with the antigen epitope recognized by the T cells. Thus, a semi-allogeneic population of T cells that can recognize at least one allelic product of the subject's MHC

molecules, preferably an HLA-DR or an HLA-DQ or other HLA molecule, and that is specific for a NS-associated antigen epitope, will be able to recognize the NS antigen in the subject's area of NS damage and produce the needed neuroprotective effect. There is little or no polymorphism in the adhesion molecules, leukocyte migration molecules, and accessory molecules needed for the T cells to migrate to the area of damage, accumulate there, and undergo activation. Thus, the semi-allogeneic T cells will be able to migrate and accumulate at the CNS site in need of neuroprotection and will be activated to produce the desired effect.

It is known that semi-allogeneic T cells will be rejected by the subject's immune system, but that rejection requires about two weeks to develop. Hence, the semi-allogeneic T cells will have the two week window of opportunity needed to exert neuroprotection. After two weeks, the semi-allogeneic T cells will be rejected from the body of the subject, but that rejection is advantageous to the subject because it will rid the subject of the foreign T cells and prevent any untoward consequences of the activated T cells. The semi-allogeneic T cells thus provide an important safety factor and are a preferred embodiment.

It is known that a relatively small number of HLA class II molecules are shared by most individuals in a population. For example, about 50% of the Jewish population express the HLA-DR5 gene. Thus, a bank of specific T cells reactive to NS antigen epitopes that are restricted to HLA-DR5 would be useful in 50% of that population. The entire population can be covered essentially by a small number of additional T cell lines restricted to a few other prevalent HLA molecules, such as DR1, DR4, DR2, etc. Thus, a functional bank of uniform T cell lines can be prepared and stored for immediate use in almost any individual in a given population. Such a bank of T cells would overcome any technical problems in obtaining a sufficient number of specific T cells from the subject in need of neuroprotection during the open window of treatment opportunity. The semi-allogeneic T cells will be safely rejected after accomplishing their role of neuroprotection. This aspect of the invention does not

contradict, and is in addition to the use of autologous T cells as described herein.

The NS-specific activated T cells are preferably non-attenuated, although attenuated NS-specific activated T cells may be used. T cells may be attenuated using methods well known in the art, including but not limited to, by gamma-irradiation, e.g., 1.5-10.0 Rads (Ben-Nun, A., Wekerle, H. and Cohen, I.R., Nature 292:60-61 (1981); Ben-Nun, A. and Cohen, I.R., J. Immunol. 129:303-308 (1982)); and/or by pressure treatment, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.); and/or by chemical cross-linking with an agent such as formaldehyde, glutaraldehyde and the like, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.); and/or by cross-linking and photoactivation with light with a photoactivatable psoralen compound, for example as described in U.S. Patent No. 5,114,721 (Cohen et al.); and/or by a cytoskeletal disrupting agent such as cytochalsin and colchicine, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.). In a preferred embodiment the NS-specific activated T cells are isolated as described below. T cells can be isolated and purified according to methods known in the art (Mor and Cohen, 1995, J. Immunol. 155:3693-3699). For an illustrative example, see Section 6.1.

Circulating T cells of a subject which recognize myelin basic protein or another NS antigen, such as the amyloid precursor protein, are isolated and expanded using known procedures. In order to obtain NS-specific activated T cells, T cells are isolated and the NS-specific ATCs are then expanded by a known procedure (Burns et al., Cell Immunol. 81:435, 1983; Pette et al., Proc. Natl. Acad. Sci. USA 87:7968, 1990; Mortin et al., J. Immunol. 145:540, 1990; Schluesener et al., J. Immunol. 135:3128, 1985; Suruhan-Dires Keneli et al., Euro. J. Immunol. 23:530, 1993, which are incorporated herein by reference in their entirety).

The isolated T cells may be activated by exposure of the cells to one or more of a variety of natural or synthetic NS-specific antigens or epitopes, including but not limited to, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated

glycoprotein (MAG), S-100, β -amyloid, Thy-1, P0, P2 and neurotransmitter receptors. In a preferred embodiment, the isolated T cells are activated by one or more cryptic epitopes, including but limited to the following MBP peptides: p11-30, p51-70, p91-110, p131-150, and p-151-170.

During *ex vivo* activation of the T cells, the T cells may be activated by culturing them in medium to which at least one suitable growth promoting factor has been added. Growth promoting factors suitable for this purpose include, without limitation, cytokines, for instance tumor necrosis factor α (TNF- α), interleukin 2 (IL-2), and interleukin 4 (IL-4).

In one embodiment, the activated T cells endogenously produce a substance that ameliorates the effects of injury or disease in the NS.

In another embodiment, the activated T cells endogenously produce a substance that stimulates other cells, including, but not limited to, transforming growth factor- β (TGF- β), nerve growth factor (NGF), neurotrophic factor 3 (NT-3), neurotrophic factor 4/5 (NT-4/5), brain derived neurotrophic factor (BDNF); interferon- γ (IFN- γ), and interleukin-6 (IL-6), wherein the other cells, directly or indirectly, ameliorate the effects of injury or disease.

Following their proliferation *in vitro*, the T cells are administered to a mammalian subject. In a preferred embodiment, the T cells are administered to a human subject. T cell expansion is preferably performed using peptides corresponding to sequences in a non-pathogenic, NS-specific, self protein.

A subject can initially be immunized with an NS-specific antigen using a non-pathogenic peptide of the self protein. A T cell preparation can be prepared from the blood of such immunized subjects, preferably from T cells selected for their specificity towards the NS-specific antigen. The selected T cells can then be stimulated to produce a T cell line specific to the self-antigen (Ben-Nun et al., J. Immunol. 129:303, 1982).

The NS-specific antigen may be a purified antigen or a crude NS preparation, as will be described below. NS-

specific antigen activated T cells, obtained as described above, can be used immediately or may be preserved for later use, e.g., by cryopreservation as described below. NS-specific activated T cells may also be obtained using previously cryopreserved T cells, i.e., after thawing the cells, the T cells may be incubated with NS-specific antigen, optimally together with thymocytes, to obtain a preparation of NS-specific ATCs.

As will be evident to those skilled in the art, the T cells can be preserved, e.g., by cryopreservation, either before or after culture.

Cryopreservation agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, Nature 183:1394-1395, 1959; Ashwood-Smith, Nature 190:1204-1205, 1961), glycerol, polyvinylpyrrolidone (Rinfret, Ann. N.Y. Acad. Sci. 85:576, 1960), polyethylene glycol (Sloviter and Ravdin, Nature 196:548, 1962), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe et al., Fed. Proc. 21:157, 1962), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender et al., J. Appl. Physiol. 15:520, 1960), amino acids (Phan The Tran and Bender, Exp. Cell Res. 20:651, 1960), methanol, acetamide, glycerol monoacetate (Lovelock, Biochem. J. 56:265, 1954), inorganic salts (Phan The Tran and Bender, Proc. Soc. Exp. Biol. Med. 104:388, 1960; Phan The Tran and Bender, 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P.L.T., ed., Butterworth, London, p. 59), and DMSO combined with hydroxyethyl starch and human serum albumin (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980).

A controlled cooling rate is critical. Different cryoprotective agents (Rapatz et al., Cryobiology 5(1):18-25, 1968) and different cell types have different optimal cooling rates. See, e.g., Rowe and Rinfret, Blood 20:636 (1962); Rowe, Cryobiology 3(1):12-18 (1966); Lewis et al., Transfusion 7(1):17-32 (1967); and Mazur, Science 168:939-949 (1970) for effects of cooling velocity on survival of cells and on their transplantation potential. The heat of fusion phase where water turns to ice should be minimal. The cooling procedure

can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure.

Programmable freezing apparatuses allow determination of optimal cooling rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planar permit tuning of the freezing regimen to the desired cooling rate curve.

After thorough freezing, cells can be rapidly transferred to a long-term cryogenic storage vessel. In one embodiment, samples can be cryogenically stored in mechanical freezers, such as freezers that maintain a temperature of about -80°C or about -20°C. In a preferred embodiment, samples can be cryogenically stored in liquid nitrogen (-196°C) or its vapor. Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators, which resemble large Thermos containers with an extremely low vacuum and internal super insulation, such that heat leakage and nitrogen losses are kept to an absolute minimum.

Considerations and procedures for the manipulation, cryopreservation, and long term storage of T cells can be found, for example, in the following references, incorporated by reference herein: Gorin, Clinics in Haematology 15(1):19-48 (1986); Bone-Marrow Conservation, Culture and Transplantation, Proceedings of a Panel, Moscow, July 22-26, 1968, International Atomic Energy Agency, Vienna, pp. 107-186.

Other methods of cryopreservation of viable cells, or modifications thereof, are available and envisioned for use, e.g., cold metal-mirror techniques. See Livesey and Linner, Nature 327:255 (1987); Linner et al., J. Histochem. Cytochem. 34(9):1123-1135 (1986); see also U.S. Patent No. 4,199,022 by Senken et al., U.S. Patent No. 3,753,357 by Schwartz, U.S. Patent No. 4,559,298 by Fahy.

Frozen cells are preferably thawed quickly (e.g., in a water bath maintained at 37-47°C) and chilled immediately upon thawing. It may be desirable to treat the cells in order to prevent cellular clumping upon thawing. To prevent clumping, various procedures can be used, including but not limited to the addition before or after freezing of DNase (Spitzer et al., Cancer 45:3075-3085, 1980), low molecular

weight dextran and citrate, citrate, hydroxyethyl starch (Stiff et al., Cryobiology 20:17-24, 1983), or acid citrate dextrose (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980), etc.

The cryoprotective agent, if toxic in humans, should be removed prior to therapeutic use of the thawed T cells. One way in which to remove the cryoprotective agent is by dilution to an insignificant concentration.

Once frozen T cells have been thawed and recovered, they are used to promote neuronal regeneration as described herein with respect to non-frozen T cells. Once thawed, the T cells may be used immediately, assuming that they were activated prior to freezing. Preferably, however, the thawed cells are cultured before injection to the patient in order to eliminate non-viable cells. Furthermore, in the course of this culturing over a period of about one to three days, an appropriate activating agent can be added so as to activate the cells, if the frozen cells were resting T cells, or to help the cells achieve a higher rate of activation if they were activated prior to freezing. Usually, time is available to allow such a culturing step prior to administration as the T cells may be administered as long as a week after injury, and possibly longer, and still maintain their neuroregenerative and neuroprotective effect.

5.2 NS-SPECIFIC ANTIGENS AND PEPTIDES DERIVED THEREFROM

Pharmaceutical compositions comprising an NS-specific antigen or peptide derived therefrom or derivative thereof can be used for preventing or inhibiting the effects of injury or disease that result in NS degeneration or for promoting nerve regeneration in the NS, particularly in the CNS. Additionally, NS-specific antigens or peptides derived therefrom or derivatives thereof may be used for *in vivo* or *in vitro* activation of T cells. In one embodiment, the NS-specific antigen is an isolated or purified antigen. In another embodiment, methods of promoting nerve regeneration or of preventing or inhibiting the effects of CNS or PNS injury or disease comprise administering NS-specific antigen or a peptide derived therefrom or derivative thereof to a mammal wherein the

NS-specific antigen or peptide derived therefrom or derivative thereof activates T cells *in vivo* to produce a population of T cells that accumulate at a site of injury or disease of the CNS or PNS.

The NS-specific antigen may be an antigen obtained from NS tissue, preferably from tissue at a site of CNS injury or disease. The NS-specific antigen may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of antigens. The functional properties may be evaluated using any suitable assay. In the practice of the invention, natural or synthetic NS-specific antigens or epitopes include, but are not limited to, MBP, MOG, PLP, MAG, S-100, β -amyloid, Thy-1, P0, P2 and a neurotransmitter receptor.

Specific illustrative examples of useful NS-specific antigens include but are not limited to, human MBP, depicted in Fig. 21, (SEQ ID NO:12); human proteolipid protein, depicted in Fig. 22 (SEQ ID NO:13); and human oligodendrocyte glycoprotein, depicted in Fig. 23 (SEQ ID NO:14).

In a preferred embodiment, peptides derived from NS-specific, self-antigens or derivatives of NS-specific antigens activate T cells, but do not induce an autoimmune disease. An example of such peptide is a peptide comprising amino acids 51-70 of myelin basic protein (residues 51-70 of SEQ ID NO:12).

In addition, an NS-specific antigen may be a crude NS-tissue preparation, e.g., derived from NS tissue obtained from mammalian NS. Such a preparation may include cells, both living or dead cells, membrane fractions of such cells or tissue, etc.

An NS-specific antigen may be obtained by an NS biopsy or necropsy from a mammal including, but not limited to, from a site of CNS injury; from cadavers; from cell lines grown in culture. Additionally, an NS-specific antigen may be a protein obtained by genetic engineering, chemically synthesized, etc.

In addition to NS-specific antigens, the invention also relates to peptides derived from NS-specific antigens or

derivatives including chemical derivatives and analogs of NS-specific antigens which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length NS-specific antigen. Such functional activities include but are not limited to antigenicity (ability to bind (or compete with an NS-antigen for binding) to an anti-NS-specific antibody), immunogenicity (ability to generate antibody which binds to an NS-specific protein), and ability to interact with T cells, resulting in activation comparable to that obtained using the corresponding full-length antigen. The crucial test is that the antigen which is used for activating the T cells causes the T cells to be capable of recognizing an antigen in the NS of the mammal (patient) being treated.

A peptide derived from a CNS-specific or PNS-specific antigen preferably has a sequence comprised within the antigen sequence and is either: (1) an immunogenic peptide, i.e., a peptide that can elicit a human T cell response detected by a T cell proliferation or by cytokine (e.g. interferon (IFN)- γ , interleukin (IL)-2, IL-4 or IL-10) production or (2) a "cryptic epitope" (also designated herein as "immunosilent" or "nonimmunodominant" epitope), i.e., a peptide that by itself can induce a T cell immune response that is not induced by the whole antigen protein (see Moalem et al., Nature Med. 5(1), 1999). Cryptic epitopes for use in the present invention include, but are not limited to, peptides of the myelin basic protein sequence: peptide p11-30, p51-70, p91-110, p131-150, and p151-170. Other peptides can be identified by their capacity to elicit a human T cell response detected by T cell proliferation or by cytokine (e.g. IFN- γ , IL-2, IL-4, or IL-10) production. Such cryptic epitopes are particularly preferred as T cells activated thereby will accumulate at the injury site, in accordance with the present invention, but are particularly weak in autoimmunity. Thus, they would be expected to have fewer side effects.

In one specific embodiment of the invention, peptides consisting of or comprising a fragment of an NS-specific antigen consisting of at least 10 (contiguous) amino acids of the NS-specific antigen are provided. In other embodiments, the

fragment consists of at least 20 contiguous amino acids or 50 contiguous amino acids of the NS-specific antigen. Derivatives of an NS-specific antigen also include but are not limited to those molecules comprising regions that are substantially homologous to the full-length antigen or fragments thereof (e.g., in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding nucleotide sequence of the full-length NS-specific antigen, under high stringency, moderate stringency, or low stringency conditions.

Computer programs for determining homology may include but are not limited to TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85(8):2444-8, 1988; Altschul et al., J. Mol. Biol. 215(3):40310, 1990; Thompson, et al., Nucleic Acids Res. 22(22):4673-80, 1994; Higgins, et al., Methods Enzymol. 266:383-402, 1996; Altschul, et al., 1990, J. Mol. Biol. 215(3):403-410, 1990).

The NS-specific antigen derivatives of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the coding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to,

chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., J. Biol. Chem. 253:6551, 1978), etc.

Manipulations may also be made at the protein level. Included within the scope of the invention are derivatives which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, derivatives of an NS-specific antigen can be chemically synthesized. For example, a peptide corresponding to a portion of an antigen which comprises the desired domain or which mediates the desired activity can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acids analogs can be introduced as a substitution or addition into the amino acid sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid; 4-aminobutyric acid, Abu; 2-amino butyric acid, γ -Abu; ϵ -Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine; norleucine; novaline; hydroxyproline; sarcosine; citrulline; cysteic acid; t-butylglycine; t-butylalanine; phenylglycine; cyclohexylalanine; β -alanine; fluoro-amino acids; designer amino acids such as β -methyl amino acids, $\text{C}\alpha$ -methyl amino acids, $\text{N}\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The functional activity of NS-specific antigens and peptides derived therefrom and derivatives thereof can be assayed by various methods known in the art, including, but not limited to, T cell proliferation assays (Mor and Cohen, J. Immunol. 155:3693-3699, 1995).

An NS-specific antigen or peptide derived therefrom or derivative thereof may be kept in solution or may be provided in a dry form, e.g. as a powder or lyophilizate, to be mixed with appropriate solution prior to use.

5.3 NUCLEOTIDE SEQUENCES ENCODING NS-ANTIGENS AND PEPTIDES DERIVED THEREFROM

Compositions comprising a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used for preventing or inhibiting the effects of injury or disease that result in CNS or PNS degeneration or for promoting nerve regeneration in the CNS or PNS. Specific illustrative examples of useful nucleotide sequences encoding NS-specific antigens or peptides derived from an NS-specific antigen, include but are not limited to nucleotide sequences encoding rat myelin basic protein (MBP) peptides, depicted in Fig. 15 (SEQ ID NO:1); human MBP, depicted in Fig. 16 (SEQ ID NO:2); human myelin PLP, depicted in Figs. 17(A-F) (SEQ ID NOS:3-8); human MOG, depicted in Fig. 18 (SEQ ID NO:9); rat PLP and variant, depicted in Fig. 19 (SEQ ID NO:10); and rat MAG, depicted in Fig. 20 (SEQ ID NO:11).

5.4 THERAPEUTIC USES

The compositions described in Sections 5.1 through 5.3 may be used to promote nerve regeneration or to prevent or inhibit secondary degeneration which may otherwise follow primary NS injury, e.g., blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke or damages caused by surgery such as tumor excision. In addition, such compositions may be used to ameliorate the effects of disease that result in a degenerative process, e.g., degeneration occurring in either grey or white matter (or both) as a result of various diseases or disorders, including, without limitation: diabetic neuropathy, senile dementias, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis (ALS), non-arteritic optic neuropathy, intervertebral disc herniation, vitamin deficiency, prion diseases such as Creutzfeldt-Jakob disease, carpal tunnel syndrome, peripheral neuropathies associated with

various diseases, including but not limited to, uremia, porphyria, hypoglycemia, Sjorgren Larsson syndrome, acute sensory neuropathy, chronic ataxic neuropathy, biliary cirrhosis, primary amyloidosis, obstructive lung diseases, acromegaly, malabsorption syndromes, polycythemia vera, IgA and IgG gammapathies, complications of various drugs (e.g., metronidazole) and toxins (e.g., alcohol or organophosphates), Charcot-Marie-Tooth disease, ataxia telangiectasia, Friedreich's ataxia, amyloid polyneuropathies, adrenomyeloneuropathy, Giant axonal neuropathy, Refsum's disease, Fabry's disease, lipoproteinemia, etc.

In a preferred embodiment, the NS-specific activated T cells, the NS-specific antigens, peptides derived therefrom, derivatives thereof or the nucleotides encoding said antigens, or peptides or any combination thereof of the present invention are used to treat diseases or disorders where promotion of nerve regeneration or prevention or inhibition of secondary neural degeneration is indicated, which are not autoimmune diseases or neoplasias. In a preferred embodiment, the compositions of the present invention are administered to a human subject.

While activated NS-specific T cells may have been used in the prior art in the course of treatment to develop tolerance to autoimmune antigens in the treatment of autoimmune diseases, or in the course of immunotherapy in the treatment of NS neoplasms, the present invention can also be used to ameliorate the degenerative process caused by autoimmune diseases or neoplasms as long as it is used in a manner not suggested by such prior art methods. Thus, for example, T cells activated by an autoimmune antigen have been suggested for use to create tolerance to the autoimmune antigen and, thus, ameliorate the autoimmune disease. Such treatment, however, would not have suggested the use of T cells directed to other NS antigens or NS antigens which will not induce tolerance to the autoimmune antigen or T cells which are administered in such a way as to avoid creation of tolerance. Similarly, for neoplasms, the effects of the present invention can be obtained without using immunotherapy processes suggested in the prior art by, for example, using an NS antigen which

does not appear in the neoplasm. T cells activated with such an antigen will still accumulate at the site of neural degeneration and facilitate inhibition of this degeneration, even though it will not serve as immunotherapy for the tumor *per se*.

5.5 FORMULATIONS AND ADMINISTRATION

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. The carriers in the pharmaceutical composition may comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatin, starch, lactose or lactose monohydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil,

oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen free water, before use.

The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized

aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

In a preferred embodiment, compositions comprising NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom, or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, are formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous or intraperitoneal administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

Pharmaceutical compositions comprising NS-specific antigen or peptide derived therefrom or derivative thereof may optionally be administered with an adjuvant, such as Incomplete Freund's Adjuvant.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

In a preferred embodiment, the pharmaceutical compositions of the invention are administered to a mammal, preferably a human, shortly after injury or detection of a degenerative lesion in the NS. The therapeutic methods of the invention may comprise administration of an NS-specific activated T cell or an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, or any combination thereof.

When using combination therapy, the NS-specific antigen may be administered before, concurrently or after administration of NS-specific activated T cells, a peptide derived from an NS-specific antigen or derivative thereof or a nucleotide sequence encoding such antigen or peptide.

In one embodiment, the compositions of the invention are administered in combination with one or more of the following (a) mononuclear phagocytes, preferably cultured monocytes (as described in PCT publication No. WO 97/09985, which is incorporated herein by reference in its entirety), that have been stimulated to enhance their capacity to promote neuronal regeneration; (b) a neurotrophic factor such as acidic fibroblast growth factor; and (c) an anti-inflammatory therapeutic substance (i.e., an anti-inflammatory steroid, such as dexamethasone or methylprednisolone, or a non-steroidal anti-inflammatory peptide, such as Thr-Lys-Pro (TKP)).

In another embodiment, mononuclear phagocyte cells according to PCT Publication No. WO 97/09985 and U.S. patent application Serial No. 09/041,280, filed March 11, 1998, are injected into the site of injury or lesion within the CNS, either concurrently, prior to, or following parenteral administration of NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide

In another embodiment, administration of NS-specific activated T cells, NS-specific antigen or peptide sequence encoding such antigen or peptide, may be administered as a single dose or may be repeated, preferably at 2 week intervals and then at successively longer intervals once a month, once a quarter, once every six months, etc. The course of treatment may last several months, several years or occasionally also through the life-time of the individual, depending on the condition or disease which is being treated. In the case of a CNS injury, the treatment may range between several days to months or even years, until the condition has stabilized and there is no or only a limited risk of development of secondary degeneration. In chronic human disease or Parkinson's disease, the therapeutic treatment in accordance with the invention may be for life.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g. gender, weight, etc.) of the individual, as well as on various other factors, e.g., whether the individual is taking other drugs, etc.

The optimal dose of the therapeutic compositions comprising NS-specific activated T cells of the invention is proportional to the number of nerve fibers affected by NS injury or disease at the site being treated. In a preferred embodiment, the dose ranges from about 5×10^6 to about 10^7 for treating a lesion affecting about 10^5 nerve fibers, such as a complete transection of a rat optic nerve, and ranges from about 10^7 to about 10^8 for treating a lesion affecting about 10^6 - 10^7 nerve fibers, such as a complete transection of a human optic nerve. As will be evident to those skilled in the art, the dose of T cells can be scaled up or down in proportion to the number of nerve fibers thought to be affected at the lesion or site of injury being treated.

5.6 ESTABLISHMENT OF AUTOLOGOUS CELL BANKS FOR T LYMPHOCYTES

To minimize secondary damage after nerve injury, patients can be treated by administering autologous or semi-allogeneic T lymphocytes sensitized to at least one appropriate NS antigen. As the window of opportunity has not yet been precisely defined, therapy should be administered as soon as possible after the primary injury to maximize the chances of success, preferably within about one week.

To bridge the gap between the time required for activation and the time needed for treatment, a bank can be established with personal vaults of autologous T lymphocytes prepared for future use for neuroprotective therapy against secondary degeneration in case of NS injury. T lymphocytes are isolated from the blood and then sensitized to a NS antigen. The cells are then frozen and suitably stored under the person's name, identity number, and blood group, in a cell bank until needed.

Additionally, autologous stem cells of the CNS can be processed and stored for potential use by an individual patient in the event of traumatic disorders of the NS such as ischemia or mechanical injury, as well as for treated neurodegenerative conditions such as Alzheimer's disease or Parkinson's disease. Alternatively, semi-allogeneic or allogeneic T cells can be stored frozen in banks for use by any individual who shares one MHC type II molecule with the source of the T cells.

The following examples illustrate certain features of the present invention but are not intended to limit the scope of the present invention.

**EXAMPLE: ACCUMULATION OF ACTIVATED T CELLS IN INJURED
OPTIC NERVE**

6.1 MATERIALS AND METHODS

6.1.1 ANIMALS

Female Lewis rats were supplied by the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, IL), matched for age (8-12 weeks) and housed four to a cage in a light and temperature-controlled room.

6.1.2 MEDIA

The T cell proliferation medium contained the following: Dulbecco's modified Eagle's medium (DMEM, Biological 15 Industries, Israel) supplemented with 2mM L-glutamine (L-Glu, Sigma, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma), penicillin (100 IU/ml; Biological Industries), streptomycin (100 μ /ml; Biological Industries), sodium pyruvate (1 mM; Biological Industries), non-essential amino acids (1 ml/100 ml; Biological Industries) and autologous rat serum 1% (vol/vol) (Mor et al., *Clin. Invest.* 85:1594, 1990). Propagation medium contained: DMEM, 2-ME, L-Glu, sodium pyruvate, non-essential amino acids and antibiotics in the same concentration as above with the addition of 10% fetal calf serum (FCS), and 10% T cell growth factor (TCGF) obtained from the supernatant of concanavalin A-stimulated spleen cells (Mor et al., *supra*, 1990).

6.1.3 ANTIGENS

Myelin basic protein (MBP) from the spinal cords of guinea pigs was prepared as described (Hirshfeld, et al., FEBS Lett. 7:317, 1970). Ovalbumin was purchased from Sigma (St. Louis, Missouri). The p51-70 of the rat 18.5kDa isoform of MBP (sequence: APKRGSGKDSHTRTTHYG) (SEQ ID NO:15) and the p277 peptide of the human hsp60 (sequence: VLGGGCALLRCPALDSLTPANED) (SEQ ID NO:16) (Elias et al., Proc. Natl. Acad. Sci. USA 88:3088-3091, 1991) were synthesized using the 9-fluorenylmethoxycarbonyl technique with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptides was analyzed by HPLC and amino acid composition.

6.1.4 T CELL LINES

T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with an antigen (described above in Section 6.1.3). The antigen was dissolved in PBS (1mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco 15 Laboratories, Detroit, Michigan). The emulsion (0.1 ml) was injected into hind foot pads of the rats. Ten days after the antigen was injected, the rats were killed and draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in proliferation medium (described above in Section 6.1.2). After incubation for 72 h at 37°C, 90% relative humidity and 7% CO₂, the cells were transferred to propagation medium (described above in Section 6.1.2). Cells were grown in propagation medium for 4-10 days before being re-exposed to antigen (10 µg/ml) in the presence of irradiated (2000 red) thymus cells (10⁷ cells/ml) in proliferation medium. The T cell lines were expanded by repeated re-exposure and propagation.

6.1.5 CRUSH INJURY OF RAT OPTIC NERVE

Crush injury of the optic nerve was performed as

previously described (Duvdevani et al., Neurol. Neurosci. 2:31-38, 1990). Briefly, rats were deeply anesthetized by i.p. injection of Rompum (xylazine, 10 mg/kg; Vitamed, Israel) and Vetaler (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa). Using a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, a moderate crush injury was inflicted on the optic nerve, 2mm from the eye (Duvdevani et al., Instructure Neurology and Neuroscience 2:31, 1990). The contralateral nerve was left undisturbed and was used as a control.

6.1.6 IMMUNOCYTOCHEMISTRY OF T CELLS

Longitudinal cryostat nerve sections (20 μm thick) were picked up onto gelatin glass slides and frozen until preparation for fluorescent staining. Sections were thawed and fixed in ethanol for 10 minutes at room temperature, washed twice with double-distilled water (ddH_2O), and incubated for 3 minutes in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20; Sigma, USA). Sections were then incubated for 1 hr at room temperature with a mouse monoclonal antibody directed against rat T cell receptor (TCR) (1:100, Hunig et al., J. Exp. Med., 169:73, 1989), in PBS containing 3% FCS and 2% BSA. After three washes with PBS containing 0.05% Tween-20, the sections were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-section to rat, human, bovine and horse serum proteins) (Jackson ImmunoResearch, West Grove, Pennsylvania) for one hour at room temperature. The sections were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane (Sigma), to inhibit quenching of fluorescence. The sections were viewed with a Zeis microscope and cells were counted. Staining in the absence of first antibody was negative.

6.2. RESULTS

Fig. 1 shows accumulation of T cells measured immunohistochemically. The number of T cells was considerably higher in injured nerves rats injected with anti-MBP, anti-OVA or anti-p277 cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with ant-MBP, anti-OVA, or anti-p277 T cells and in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

EXAMPLE: NEURPROTECTION BY AUTOIMMUNE ANTI-MBP T CELLS

7.1 MATERIAL AND METHODS

Animals, media, antigens, crush injury of rat optic nerve, sectioning of nerves, T cell lines, and immunolabeling of nerve sections are described in Section 6, *supra*.

7.1.1. RETROGRADE LABELING AND MEASUREMENT OF PRIMARY DAMAGE AND SECONDARY DEGENERATION

Primary damage of the optic nerve axons and their attached retinal ganglion cells (RGCs) were measured after the immediate post-injury application of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-Asp) (Molecular Probes Europe BV, Netherlands) distal to the site of injury. Only axons that are intact are capable of transporting the dye back to their cell bodies; therefore, the number of labeled cell bodies is a measure of the number of axons that survived the primary damage. Secondary degeneration was also measured by application of the dye distal to the injury site, but two weeks after the primary lesion was inflicted. Application of the neurotracer dye distal to the site of the primary crush after two weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell

bodies are still viable, as only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled ganglion cells reliably reflects the number of still-functioning neurons. Labeling and measurement were done by exposing the right optic nerve for a second time, again without damaging the retinal blood supply. Complete axotomy was done 1-2 mm from the distal border of the injury site and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Uninjured optic nerves were similarly labeled at approximately the same distance from the globe. Five days after dye application, the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution and examined for labeled ganglion cells by fluorescence microscopy. The percentage of RGCs surviving secondary degeneration was calculated using the following formula: (Number of spared neurons after secondary degeneration) / (Number of spared neurons after primary damage) x 100.

7.1.2 ELECTROPHYSIOLOGICAL RECORDINGS

Nerves were excised and their compound action potentials (CAPs) were recorded *in vitro* using a suction electrode experimental set-up (Yoles et al., *J. Neurotrauma* 13:49-57, 1996). At different times after injury and injection of T cells or PBS, rats were killed by intraperitoneal injection of pentobarbitone (170 mg/kg) (CTS Chemical Industries, Israel). Both optic nerves were removed while still attached to the optic chiasma, and were immediately transferred to a vial containing a fresh salt solution consisting of 126 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2 mM MgSO₄, 2 mM CaCl₂ and 10 mM D-glucose, aerated with 95% O₂ and 5% CO₂ at room temperature. After 1 hour, electrophysiological recordings were made. In the injured nerve, recordings were made in a segment distal to the injury site. This segment contains axons of viable retinal ganglion cells that have escaped both primary and secondary damage, as well as the distal stumps of non-viable retinal ganglion cells

that have not yet undergone Wallerian degeneration. The nerve ends were connected to two suction Ag-AgCl electrodes immersed in the bathing solution at 37°C. A stimulating pulse was applied through the electrode, and the CAP was recorded by the distal electrode. A stimulator (SD9; Grass Medical Instruments, Quincy, Massachusetts) was used for supramaximal electrical stimulation at a rate of 1 pps to ensure stimulation of all propagating axons in the nerve. The measured signal was transmitted to a microelectrode AC amplifier (model 1800; A-M Systems, Everett, Washington). The data were processed using the LabView 2.1.1 data acquisition and management system (National Instruments, Austin, Texas). For each nerve, the difference between the peak amplitude and the mean plateau of eight CAPs was computed and was considered as proportional to the number of propagating axons in the optic nerve. The experiments were done by experimentors "blinded", to sample identity. In each experiment the data were normalized relative to the mean CAP of the uninjured nerves from PBS-injected rats,

7.1.3 CLINICAL EVALUATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Clinical disease was scored every 1 to 2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

7.2 RESULTS

7.2.1 NEUROPROTECTION BY AUTOIMMUNE anti-MBP T CELLS

Morphological analyses were done to assess the effect of the T cells on the response of the nerve to injury, and specifically on secondary degeneration. Rats were injected intraperitoneally immediately after optic nerve injury with PBS or with 1×10^7 activated T cells of the various cell lines. The degree of primary damage to the optic nerve axons and their attached RGCs was measured by injecting the dye 4-Di-10-Asp

distal to the site of the lesion immediately after the injury. A time lapse of 2 weeks between a moderate crush injury and dye application is optimal for demonstrating the number of still viable labeled neurons as a measure of secondary degeneration, and as the response of secondary degeneration to treatment. Therefore, secondary degeneration was quantified by injecting the dye immediately or 2 weeks after the primary injury, and calculating the additional loss of RGCs between the first and the second injections of the dye. The percentage of RGCs that had survived secondary degeneration was then calculated. The percentage of labeled RGCs (reflecting still-viable neurons) was significantly greater in the retinas of the rats injected with anti-MBP T cells than in the retinas of the PBS-injected control rats (Fig. 2). In contrast, the percentage of labeled 30 RGCs in the retinas of the rats injected with anti-OVA or anti-p277 T cells was not significantly greater than that in the control retinas. Thus, although the three T cell lines accumulated at the site of injury, only the MBP-specific autoimmune T cells had a substantial effect in limiting the extend of secondary degeneration. Labeled RGCs of injured optic nerves of rats injected with PBS (Fig. 3A), with anti-p277 T cells (Fig. 3B) or with anti-MBP T cells (FIG. 3C) were compared morphologically using micrographs.

7.2.2 CLINICAL SEVERITY OF EAE

Animals were injected i.p. with 10^7 T_{MBP} cells with or without concurrent optic nerve crush injury. The clinical course of the rats injected with the T_{MBP} cells was evaluated according to the neurological paralysis scale. Each group contained 5-9 rats. The functional autoimmunity of the injected anti-MBP T cells was demonstrated by the development of transient EAE in the recipients of these cells. As can be seen in Fig. 4A, the course and severity of the EAE was not affected by the presence of the optic nerve crush injury.

7.2.3 SURVIVAL OF RGCS IN NON-INJURED NERVES

Animals were injected i.p. with 10^7 T_{MBP} cells or PBS. Two weeks later, 4-Di-10-Asp was applied to the optic nerves. After five days the retinal were excised and flat

mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk), in each retina were counted and their average number per area (mm^2) was calculated.

As can be seen in Fig. 4B, there is no difference in the number of surviving RGCs per area (mm^2) in non-injured optic nerves of rats injected with anti-MBP T cells compared to in rats injected with PBS.

7.2.4. NEUROPROTECTION BY T CELLS REACTIVE TO A CRYPTIC EPITOPE

To determine whether the neuroprotective effect of the anti-MBP T cells is correlated with their virulence, the effect of T cells reactive to a "cryptic" epitope of MBP, the peptide 51-70 (p51-70) was examined. "Cryptic" epitopes activate specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen (Mor et al., *J. Immunol.* 155:3693-3699. 1995). The T cell line reactive to the whole MBP and the T cell line reactive to the cryptic epitope p51-70 were compared for the severity of the EAE they induced, and for their effects on secondary degeneration. In rats injected with the T cell line reactive to the cryptic epitope, disease severity (as manifested by the maximal EAE score) was significantly lower than that in rats injected with the T cell line reactive to the whole protein (Table 1). Whereas anti-MBP T cells caused clinical paralysis of the limbs, rats injected with the anti-p51-70 T cells developed only tail atony, not hind limb paralysis, and almost none showed weakness of the hind limbs. Despite this difference in EAE severity, the neuroprotective effect of the less virulent (anti-p51-70) T cells was similar to that of the more virulent (anti-MBP) T cells (Fig. 5). The percentage of RGCs surviving secondary degeneration in the retinas of rats injected with either of the lines was significantly higher than in the retinas of the PBS-injected rats. Thus, there was no correlation between the neuroprotective effect of the autoimmune T cells and their virulence. It is possible that the anti-p51-70 T cells encounter little antigen in the intact CNS, and therefore cause only mild EAE. Their target antigen

may however become more available after injury, enabling these T cells to exert a neuroprotective effect.

TABLE 1. Anti-MBP and anti-p51-70 T cells Vary in Pathogenicity

T Cell Line	Clinical EAE	Mean Max. Score
Whole MBP	Moderate to severe	2.00 + 0.2
p51-70 of MBP	Mild	0.70 + 0.2

Immediately after optic nerve crush injury, Lewis rats were injected with activated anti-MBP T cells or anti-p51-70 T cells. The clinical course of EAE was evaluated according to the neurological paralysis scale. The mean maximal (max.) score \pm s.e.m. was calculated as the average maximal score of all the diseased rats in each group. The table is a summary of nine experiments. Each group contains five to ten rats. Statistical analysis showed a significant difference between the mean maximal score of rats injected with anti-MBP T cells and that of rats injected with anti-p51-70 T cells ($P=0.039$, Student's t-test).

7.2.5 ELECTROPHYSIOLOGICAL ACTIVITY

To confirm the neuroprotective effect of the anti-MBP T cells, electrophysiological studies were done. Immediately after optic nerve injury, the rats were injected intraperitoneally with PBS or with 1×10^7 activated anti-MBP or anti-OVA T cells. The optic nerves were excised 7, 11 or 14 days later and the compound action potentials (CAPs), a measure of nerve conduction, were recorded from the injured nerves. On day 14, the mean CAP amplitudes of the distal segments recorded from the injured nerves obtained from the PBS-injected control rats were 33% to 50% of those recorded from the rats injected with the anti-MBP T cells (Fig. 6A, Table 2). As the distal segment of the injured nerve contains both neurons that escaped the primary insult and injured neurons that have not yet degenerated, the observed neuroprotective effect could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of the injured neurons (which normally occurs in the distal stump), or both. No effect of the injection anti-MBP T cells on the mean CAP amplitudes of uninjured nerves was

observed (Fig. 6B, Table 2). It is unlikely that the neuroprotective effect observed on day 14 could have been due to the regrowth of nerve fibers, as the time period was too short for this.

The strong neuroprotective effect of the anti-MBP T cells seen on day 14 was associated with a significantly decreased CAP amplitude recorded on day 7 (Table 2). The anti-MBP T cells manifested no substantial effect on the uninjured nerve on day 7, indicating that the reduction in electrophysiological activity observed in the injured nerve on day 7 might reflect the larger number of T cells present at the injury site relative to the uninjured nerve (Fig. 1). The observed reduction in CAP amplitude in the injured nerve on day 7 reflected a transient resting state in the injured nerve. This transient effect has not only disappeared, but was even reversed by day 14 (Table 2). Early signs of the neuroprotective effect could already be detected on day 11 in the rats injected with anti-OVA T cells, no reduction in CAP amplitude on day 7 could be detected in either the injured or the uninjured nerves, and no neuroprotective effect was observed on day 14 (Table 2). Thus, it seems that the early reduction in CAP and the late neuroprotection shown specifically by the anti-MBP T cells are related.

TABLE 2. Transient reduction in electrophysiological activity of the injured optic nerve induced by anti-MBP T cells, followed by a neuroprotective effect

	<u>Uninjured Optic Nerve</u>	<u>Injured Optic Nerve</u>		
	<u>Day 7</u>	<u>Day 14</u>	<u>Day 7</u>	<u>Day 14</u>
Ratio (%) T_{MBP}/PBS	89.9 ± 9.4 (n=22)	101.2 ± 22.7 (n=10)	$63.8^* \pm 14.9$ (n=17)	$243.1^{**} \pm 70.8$ (n=8)
Ratio (%) T_{OVA}/PBS	109.7 ± 13.2 (n=11)	92.5 ± 12.6 (n=3)	125.5 ± 24.4 (n=11)	107.3 ± 38.9 (n=4)

Immediately after optic nerve injury, rats were injected with PBS or with activated anti-MBP or anti-OVA T cells. After 7 or 14 days, the CAPs of injured and uninjured nerves were recorded. Ratios were calculated for uninjured nerves as (mean CAP of uninjured nerves from T cell-injected rats/mean CAP of uninjured nerves from PBS-injected rats) x 100, or for injured

nerves as (mean CAP of injured nerves from T cell-injected rats/mean CAP of injured nerves from PBS-injected rats) x 100. The P value was calculated by comparing the logarithms of the normalized CAP amplitudes of nerves from PBS-injected rats and rats injected with T cells, using the unpaired Student's test, * P<0.05; ** P<0.001 n=sample size.

7.3 NEUROPROTECTION IN SPINAL CORD INJURY

7.3.1. MATERIALS AND METHODS

Animals, antigens (MBP, OVA) and T cell lines were as described hereinbefore in 6.1.1, 6.1.3 and 6.1.4, respectively

Contusion. Adult rats (300 to 350g) were anesthetized and the spinal cord was exposed by laminectomy at the level of T7-T8. One hour after induction of anesthesia, a 10 gram rod was dropped onto the laminectomized cord from a height of 50 mm. The impactor device (designed by Prof. Wise Young) allowed, for each animal, measurement of the trajectory of the rod and its contact with the spinal cord to allow uniform lesion. Within an hour of the contusion, rats were injected i.p., on a random basis, with either 10^7 cells (specific to either MBP or OVA, depending on the experimental design) or with PBS. Bladder expression was done at least twice a day (particularly during the first 48h after injury, when it was done 3 times a day) until the end of the second week, by which time the rats had developed autonomous bladder voidance. Approximately twice a week, locomotor activity (of the trunk, tail and hind limbs) in an open field was evaluated by placing the rat for 4 min in the middle of a circular enclosure made of molded plastic with a smooth, non-slip floor (90 cm diameter, 7 cm wall height).

7.3.2 RESULTS

The present study of spinal cord neuroprotection was prompted by the previous example that partial injury to an optic nerve can be ameliorated administering T cells directed to a CNS self-antigen. The question was whether autoimmune T cells could have a beneficial effect on recovery from traumatic spinal cord injury with its greater mass of injured CNS tissue and the attendant spinal shock.

Adult Lewis rats were subjected to a calibrated spinal cord contusion produced by dropping a 10 gram weight from a height of 50 mm onto the laminectomized cord at the level of T7-T8 (see description included in Basso et al., Exp-Neurol 139, 244-256, 1996). The rats were then injected intraperitoneally with autoimmune T cells specific to MBP. Control rats were similarly injured but received either no T cells or T cells specific to the non-self antigen ovalbumin (OVA). Recovery of the rats was assessed every 3 to 4 days in terms of their behavior in an open-field locomotion test, in which scores range from 0 (complete paraplegia) to 21 (normal mobility). The locomotor performance of the rats was judged by observers blinded to the identity of the treatment received by the rats. Included in the study was a group of uninjured, sham-operated (laminectomized but not contused) rats which were injected with anti-MBP T cells to verify the activity of the T cells. In all the sham-operated rats, the anti-MBP T cells induced clinical experimental autoimmune encephalomyelitis (EAE), which developed by day 4, reached a peak at day 7 and resolved spontaneously by day 11. Note, therefore, that at the early post-traumatic stage, any effect of the autoimmune T cells on the injured spinal cord, whether positive or negative, would be transiently masked both by spinal shock and by the paralysis of EAE.

Indeed, none of the rats with contused spinal cords showed any locomotor activity in the first few days after the contusion (Fig. 7A). Interestingly, however, the rats treated with anti-MBP T cells recovered earlier from spinal shock; on day 11, for example, when no recovery could be detected in any of the untreated control rats, significant improvement was noted in the T cell-treated rats (Fig. 7A). At all time points thereafter, the rats that had received the autoimmune T cells showed better locomotor recovery than did the untreated injured rats (Fig. 7A). Thus the autoimmune T cells, in spite of being encephalitogenic, did confer significant neuroprotection. Moreover, the phase of neuroprotective activity coincided with the phase of immune paralysis, supporting our suggestion that neuroprotection might be related to transient paralysis.

By one month after trauma the rats in both groups had reached a maximal behavioral score, which then remained at plateau for at least 3 months of follow-up. In the untreated rats, maximal recovery of locomotor behavior, as noted in previous reports of similarly severe contusion (Basso et al., *supra*), was marked by some ineffectual movement of hind-limb joints, but the rats showed no ability support their body weight and walk, and obtained a score of 7.3 ± 0.8 (mean \pm SEM). In contrast, the average score of the rats that had been treated with the anti-MBP T cells was 10.2 ± 0.8 , and in some rats the value was high as 13. All the rats in the treated group could support their body weight and some could frequently walk in a coordinated fashion. The difference between the two groups, based on 2-factor repeated ANOVA, was statistically significant ($p<0.05$). The recovery curve based on locomotor activity is nonlinear. The above-described increase in motor activity seen after treatment with the anti-MBP T cells could result from much higher percentage of spared tissue based on a linear regression curve on which the behavioral score is correlated with the amount of neural spinal cord tissue (for example, a difference between 11 and 7) on the locomotion score would be read as a difference between 30% and less than 10% of spared tissue).

In another set of experiments the rats were subjected to a more severe insult, resulting in a functional score of 1.9 ± 0.8 (mean \pm SEM) in the untreated group and 7.7 ± 1.4 in the treated group (Fig. 7B). This difference of more than 3 fold in behavioral scores was manifested by the almost total lack of motor activity in the control rats as compared with the ability of the autoimmune T cell-treated rats to move all their joints. The beneficial effect was specific to treatment with anti-MBP T cells; no effect was observed after treatment with T cells specific to the non-self antigen OVA (data not shown). The positive effect of the autoimmune T cells seems to be expressed in the preservation of CNS tissue that escaped the initial lesion, i.e., in neuroprotection. Therefore, the magnitude of the effect would be inherently limited by the severity of the insult; the more severe the lesion, the less the amount of spared tissue amenable to neuroprotection.

To determine whether clinical recovery could be explained in terms of preservation of spinal axons, we performed retrograde labeling of the descending spinal tracts by applying the dye rhodamine dextran amine (Brandt et al, J-Neurosci-Methods 45:35-40, 1992) at T12, below the site of damage. The number of dye-stained cells that could be counted in the red nucleus of the brain constituted a quantitative measure of the number of intact axons traversing the area of contusion. Sections of red nuclei from injured rats treated with anti-MBP T cells (Fig. 8) contained 5-fold more labeled cells than sections taken from the untreated injured rats. Photomicrographs of red nuclei taken from rats treated with anti-MBP T cells (with an open field score of 10) and from PBS-treated rats (with a score of 6) are shown in Fig. 8. These findings indicate that the reduction in injury-induced functional deficit observed in the T cell-treated rats can be attributed to the sparing of spinal tracts, resulting in a higher degree of neuron viability.

After a follow-up of more than 3 months, when the locomotor activity scores had reached a plateau, the site of injury of three of PBS-treated animals and three animals treated with anti-MBP T cells were analyzed by diffusion-weighted MRI. The cords were excised in one piece from top to bottom and were immediately placed in fixative (4% paraformaldehyde). Axial sections along the excised contused cord were analyzed. Fig. 9 shows the diffusion anisotropy in axial sections along the contused cord of a rat treated with autoimmune T cells, as compared with that of PBS-treated control rat. The images show anisotropy in the white matter surrounding the grey matter in the center of the cord. Sections taken from the lesion sites of PBS-treated control rats show limited areas of anisotropy, which were significantly smaller than those seen at comparable sites in the cords of the rats treated with the anti-MBP T cells. Quantitative analysis of the anisotropy, reflecting the number of spared fibers, is shown in Fig. 9. The imaging results show unequivocally that, as a result of the treatment with the autoimmune anti-MBP T cells, some spinal cord tracts had escaped the degeneration that would otherwise have occurred.

7.3.3 DISCUSSION OF RESULTS

No cure has yet been found for spinal cord lesions, one of the most common yet devastating traumatic injuries in industrial societies. It has been known for more than 40 years that CNS neurons, unlike neurons of the peripheral nervous system, possess only a limited ability to regenerate after injury. During the last two decades, attempts to promote regeneration have yielded approaches that lead to partial recovery. In the last few years it has become apparent that, although most of the traumatic injuries sustained by the human spinal cord are partial, the resulting functional loss is nevertheless far worse than could be accounted for by the severity of the initial insult; the self-propagating process of secondary degeneration appears to be decisive.

A substantial research effort has recently been directed to arresting injury-induced secondary degeneration. All attempts up to now have been pharmacologically based, and some have resulted in improved recovery from spinal shock. The present study, in contrast, describes a cell therapy that augments what seems to be a natural mechanism of self-maintenance and leads, after a single treatment, to long-lasting recovery. The extent of this recovery appears to exceed that reported using pharmacological methods.

In most tissues, injury-induced damage triggers a cellular immune response that acts to protect the tissue and preserve its homeostasis. This response has been attributed to macrophages and other cells comprising the innate arm of the immune system. Lymphocytes, which are responsible for adaptive immunity, have not been thought to participate in tissue maintenance. Adaptive immunity, according to traditional teaching, is directed against foreign dangers. Our studies now show, however, that the adaptive T cell immune response can be protective even when there is no invasion by foreign pathogens. In the case of tissue maintenance, the specificity of the T cells is to tissue self-antigens.

Our observation of post-traumatic CNS maintenance by autoimmune T cells suggests that we might do well to reevaluate some basic concepts of autoimmunity. T cells that are specific

to CNS self antigens in general, and to MBP in particular, have long been considered to be only detrimental to health. In the present study, however, the same T cell preparation that can produce EAE in the undamaged CNS was found to be neuroprotective in the damaged spinal cord, suggesting that the context of the tissue plays an important part in determining the outcome of its interaction with T cells. It would seem that the tissue deploys specific signals to elicit particular T cell behaviors. Among such signals are costimulatory molecules, particularly members of the B7 family (Lenchow et al., Annu. Rev. Immunol. 14:233-258, 1996). As shown hereinafter, the injured rat optic nerve transiently expresses elevated levels of the costimulatory molecule B7.2, which is constitutively expressed at low levels in the rat CNS white matter and which is thought to be associated with regulation of the cytokine profile of the responding T cells (H. L. Weiner, Annu. Rev. Med. 48:341-51, 1997). The early post-injury availability of the exogenous anti-MBP T cells, coinciding with the observed early post-injury increase in B7.2 would support the idea that signals expressed by the tissue might modulate the T cell response. It is thus conceivable that anti-MBP T cells which cause a monophasic autoimmune disease upon interacting with a healthy CNS nerve, might implement a maintenance program when they interact with damaged CNS tissue expressing increased amounts of B7.2 and probably other costimulatory molecules. The neuroprotective effects of the T cells may be mediated, at least in part, by antigen-dependent regulation of specific cytokines or neurotrophic factors (M. Kerschensteiner et al., J. Exp. Med. 189:865-870, 1999) produced locally at the site of injury.

Thus, the present invention is also directed to manipulating B7.2 co-stimulatory molecule to prevent or inhibit neuronal degeneration and ameliorate the effects of injury to or disease of the nervous system. B7.2 molecule can be up-regulated for this purpose, using drugs or by genetic manipulation, without undue experimentation.

In a recent study, it was reported that injury to the spinal cord triggers a transient autoimmune response to MBP (Popovich et al., J. Neurosci. Res. 45:349-63, 1996). However,

whether that response is detrimental or beneficial remained an open question (Popovich et al, *J. Comp. Neurol.* 377:443-464, 1997). From our present data, it would appear that the activation of anti-MBP T cells could indeed be beneficial. However, a supplement of exogenous autoimmune T cells may be required to overcome the restrictions on immune reactivity imposed by the immune-privilege of the CNS (J. W. Streilein, *Science* 270:1158-1159, 1995). The finding that autoimmune response can be advantageous suggests that natural autoimmune T cells may have undergone positive selection during ontogeny, as proposed by the theory of the immunological homunculus (I. R. Cohen, *Immunol. Today* 13, 490-494 (1992), and are not merely a default resulting from the escape from negative selection of T cells that recognize self antigens (C. A. Janeway, Jr., *Immunol. Today* 13:11-6, 1992). Such a response could then be considered as a mechanism of potential physiological CNS self-maintenance, which is, however, not sufficient for the purpose because of the immune-privileged character of the CNS.

A single injection of autoimmune T cells lasted for at least 100 days. Thus, this procedure offers a form of self-maintenance. This specific autoimmune response, when properly controlled, is useful as part of a self-derived remedy for spinal cord injury.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF NS-SPECIFIC ANTIGEN

8.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling are described above in Sections 6 and 7. A peptide based on amino acids 35-55 of myelin/oligodendrocyte glycoprotein (MOG p35-55) was chemically synthesized at the Weizmann Institute, Israel.

8.1.1 INHIBITION OF SECONDARY DEGENERATION

Rats were injected intradermally in the footpads with MOG p35-55 (50 µg/animal) and IFA, or PBS ten days prior to optic nerve crush injury. Retinal ganglion cells were assessed two weeks after injury using retrograde labeling as described above. The number of RGCs in rats injected with PBS or MOG

p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

8.2 RESULTS

As shown in Fig. 10, the number of labeled retinal ganglion cells (indicating viable axons) was about 12.5 fold greater in animals injected with MOG p35-55 compared to animals receiving PBS.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF MBP ADMINISTERED ORALLY

9.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling of RGCs are described above in Sections 6 and 7.

9.1.1 INHIBITION OF SECONDARY DEGENERATION

Bovine MBP (Sigma, Israel) (1 mg/dose) was administered to rats by gavage using a blunt needle. MBP was administered 5 times, every third day, beginning 2 weeks prior to optic nerve crush injury. The number of RGCs in treated animals was expressed as a percentage of the total number of neurons in animals subjected to optic nerve crush injury but which did not receive MBP.

9.2 RESULTS

As shown in Fig. 11, the number of labeled RGCs was about 1.3 fold greater in animals treated with MBP compared to untreated animals.

9.3 THE B7.2 COSTIMULATORY MOLECULE IS ASSOCIATED WITH POST-TRAUMATIC MAINTENANCE OF THE OPTIC NERVE BY ORAL ADMINISTRATION OF MBP

9.3.1 INTRODUCTION

Autoimmune T cells can under certain conditions be beneficial to traumatized CNS axons. The effect of such T cells on the damaged tissue might be influenced by the nature

and amount of the costimulatory molecules it expresses. We show that the B7.2 costimulatory molecule is constitutively expressed in the intact rat optic nerve, and after injury is up-regulated at the margins of the injury site. Pre-injury induction of oral tolerance to MBP resulted in a further post-injury increase in B7.2 at the margins and at the injury site itself, as well as a better preservation of the traumatized nerve. Thus, B7.2 expression in the brain and its up-regulated after trauma seem to be directly related to post-traumatic maintenance displayed by autoimmune T cells.

Neuronal injury in the CNS causes degeneration of directly damaged fibers as well as of fibers that escaped the primary insult. It also triggers a systemic response of autoimmune T cells to MBP, that might affect the course of degeneration of the injured nerve. Whether the effect of these T cells on the nerve is detrimental or beneficial may depend, in part, on the nature and level of the costimulatory molecules expressed by the damaged tissue. Several costimulatory molecules have recently been identified, including the B7 and CD40 molecules (Caux et al., "Activation of Human Dendritic Cells Through CD40 Cross-Linking", J. Exp. Med. 180:1263-1272, 1994; and Lenschow et al., "CD28/B7 System of T Cell Costimulation", Annu. Rev. Immunol. 14:233-258, 1996). CD40 appears to be dominant during cell differentiation in the lymph nodes and B7 during activation of T cells in the target organ (Grewal et al., "Requirement for CD40 Ligand in Costimulation Induction, T Cell Activation, and Experimental Allergic Encephalomyelitis", Science 273:1864-1867, 1996). B7 costimulatory molecules are expressed on antigen-presenting cells (APCs) as B7.1 or B7.2., which might preferentially support activation of the Th1 or the Th2 type of immune response, respectively (Kuchroo et al., "B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy", Cell 80:707-718, 1995; and Karandikar et al., "Targeting the B7/CD28:CTLA-4 costimulatory system in CNS autoimmune disease", J. Neuroimmunol. 89:10-18, 1998). We were therefor interested in determining the identity B7 subtype expressed in intact and injured CNS white matter, and its

possible influence on the course of the response to the injury.

9.3.2 RESULTS

The costimulatory molecule expressed constitutively in the intact optic nerves of adult Lewis rats was identified as B7.2. (Figs. 12A, 12B). To examine the effects of neurotrauma on the expression of B7 costimulatory molecules, we inflicted a mild crush injury on the optic nerves of Lewis rats and assessed the neural expression of B7 by immunohistochemical analysis. The most striking effect of the injury was seen on B7.2 expression manifested on post-injury day 3 by its elevation at the margins of the injury site (Figs. 12C,D,E). In contrast, expression of B7.1 was not detected in the optic nerve either before or 3 days after injury. On day 7, however, B7.1 was detectable at the site of injury, having pattern reminiscent of that seen for macrophages or microglia (Fig. 12F).

Next, we attempted to determine whether the degenerative response to optic nerve injury could be modified by peripheral manipulation of the immune system. The manipulation chosen was induction of oral tolerance, known to cause a "bystander" T cell immunosuppressive effect (Weiner et al., "Tolerance Immune Mechanisms and Treatment of Autoimmune Diseases", Immunol. Today 18:335-343, 1997). Ingestion of low doses of MBP results in the activation of T cells which, based on antigen recognition, secrete TGF as the dominant cytokine and thus favor an immune response of Th2/3 type (Chen, Y., "Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis", Science 265: 1237-1240, 1994).

Lewis rats were fed with food to which 1 mg of bovine MBP had been added five times daily every other day. Ten days after first receiving the supplement, the rats were subjected to mild unilateral optic nerve crush injury. This time interval between initiation of oral tolerance and injury was chosen to allow adequate build-up of the systemic T cell response. As shown in Fig. 13A and B, the numbers of macrophages or active microglia (indicated by ED-1 labeling)

and T cells (indicated by immunolabeling for T cell receptor), assessed 3 days after injury, did not differ from those observed in control injured rats which did receive any treatment or were fed with PBS. In the rats with induced oral tolerance to MBP, however, the amounts B7.2 were further increased at the margins of the site of injury (Fig. 13C) as compared with controls (Fig. 12E). In addition, B7.2 in the rats with induced oral tolerance to MBP was also elevated at the site of injury relative to the control nerves (Fig. 13C). It seems reasonable to assume that the T cells exposed to MBP via intestinal absorption, upon invading the injured CNS, contributed to the increase in expression of B7.2 by the injured nerve.

We then attempted to determine whether the observed changes in B7.2 expression in the injured rats was correlated with the extent of neuronal degeneration. Acute injury of the rat optic nerve is followed by a process of nerve degeneration, which can be quantified by retrograde labeling of the surviving neurons and counting of the corresponding cell bodies. Two weeks after optic nerve injury the number of surviving retinal ganglion cells (RGCs), representing still-viable neurons, in the group of MBP-fed rats was significantly higher than that in the control group, or than in the group of rats with injured nerves that were fed with ovalbumin. Interestingly, the benefit of the induced oral tolerance to MBP was increased by feeding the rats with more intensive schedule (Fig. 14).

DISCUSSION OF EXPERIMENTAL RESULTS

The results of the experiments described in Sections 6 and 7 show that activated T cells accumulate at a site of injury in the CNS. Furthermore, the results also demonstrate that the accumulation of T cells at the site of injury is a non-specific process, i.e., T cells which accumulated at the site of injury included both T cells which are activated by exposure to an antigen present at the site of injury as well as T cells which are activated by an antigen not normally present in the individual.

The results of experiments described in Section 7 demonstrate that the beneficial effects of T cells in

ameliorating damage due to injury in the CNS are associated with an NS-specific self-antigen as illustrated by MBP. More specifically, the administration of non-recombinant T cells which were activated by exposure to an antigen which can cause autoimmune disease (T_{MBP}), rather than aggravating the injury, led to a significant degree of protection from secondary degeneration. Thus, activating T cells by exposure to a fragment of an NS-specific antigen was beneficial in limiting the spread of injury in the CNS. The present findings show that secondary degeneration can be inhibited by the transfer into the individual on non-recombinant T cells which recognize an NS-specific self antigen which is present at a site of injury. The T cells may recognize cryptic or non-pathogenic epitopes of NS-self antigens.

In addition, the studies described in Sections and 9 show that activation of T cells by administering an immunogenic antigen (e.g. MBP) or immunogenic epitope of an antigen (e.g. MOG p35-55), may be used for preventing or inhibiting secondary CNS degeneration following injury.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to

the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same function can be used; and it is intended that such expressions be given their broadest interpretation.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A composition for preventing or inhibiting degeneration in the central nervous system or peripheral nervous system for ameliorating the effects injury or disease, comprising:

- (a) NS-specific activated T cells;
- (b) NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or
- (f) any combination of (a)-(e).

2. A composition according to claim 1, for promoting nerve regeneration in the central nervous system or peripheral nervous system for ameliorating the effects of injury or disease.

3. The composition of claim 1 or 2 in which said injury comprises spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, or ischemic stroke.

4. The composition of claim 1 or 2 in which said disease is Diabetic neuropathy, senile dementia, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis, non-arteritic optic neuropathy, or vitamin deficiency.

5. The composition of claim 1 or 2 in which said disease is not an autoimmune disease or a neoplasm.

6. The composition according to any of claims 1-5 wherein said NS-specific activate T cells of (a) are autologous T cells, or allogeneic T cells from related donors, OR HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

7. The composition according to claim 6 wherein said autologous T cells have been stored or are derived from autologous CNS cells.

8. The composition according to claim 6 wherein said T cells are semi-allogeneic T cells.

9. The composition according to any of claims 1-5 wherein said NS-specific antigen of (b) is elected from myelin basic protein (MBP), myelin oligodenrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), S-100, β -amyloid, Thy-1, P0, P2 and neurotransmitter receptors.

10. The composition according to any one of claims 1-5 wherein said peptide derived from an NS-specific antigen is an immunogenic epitope or a cryptic epitope of said antigen.

11. The composition according to claim 10 wherein said peptide is an immunogenic epitope or a cryptic epitope derived from MBP.

12. The composition according to claim 11 wherein said peptide corresponds to the sequences p11, p51-70, p91-110, p131-150, or p151-170 of MBP.

13. The compositions according to any one of claims 1-5 and 11-12 in which said NS-specific antigen or a peptide derived therefrom is administered intravenously, orally, intranasally, intrathecally, intramuscularly, intradermally, topically, subcutaneously, mucosally (e.g., orally, intranasally, vaginally, rectally) or buccally.

14. The composition according to claim 13 comprising MBP for oral administration.

15. Use of:

- (a) NS-specific activated T cells;
- (b) an NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or
- (f) any combination of (a)-(e),

for the preparation of a composition for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system for ameliorating the effects of injury or disease.

16. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral

nervous system, which comprises administering to an individual in need thereof an effective amount of:

- (a) NS-specific activated T cells;
- (b) NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or
- (f) any combination of (a)-(e).

17. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition according to any one of claims 1-13 and actively immunizing said individual to build up a critical T cell response.

18. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition for up-regulating B7.2 costimulatory molecule or genetically manipulating B7.2 costimulatory molecule in said individual.

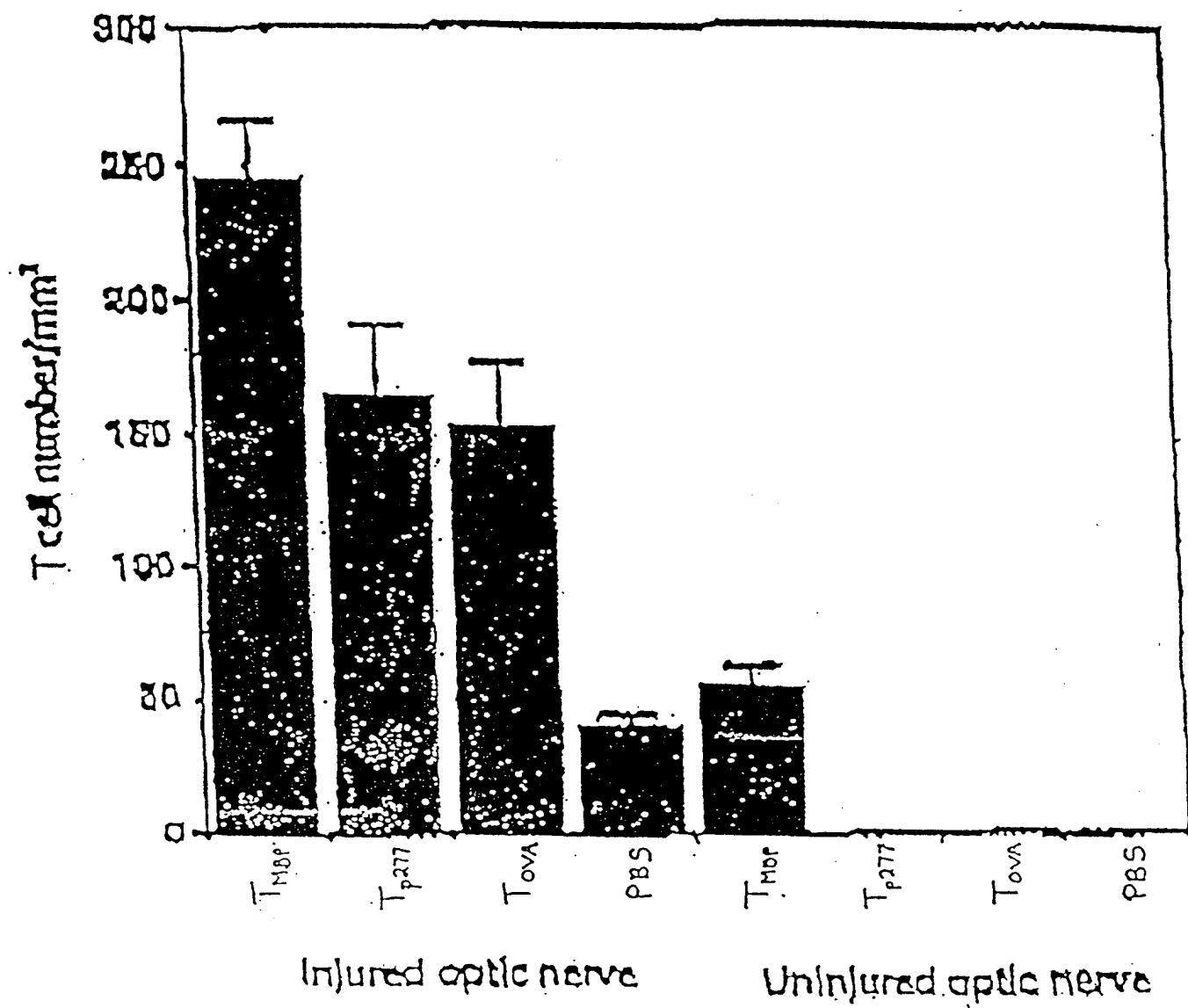


FIG. 1

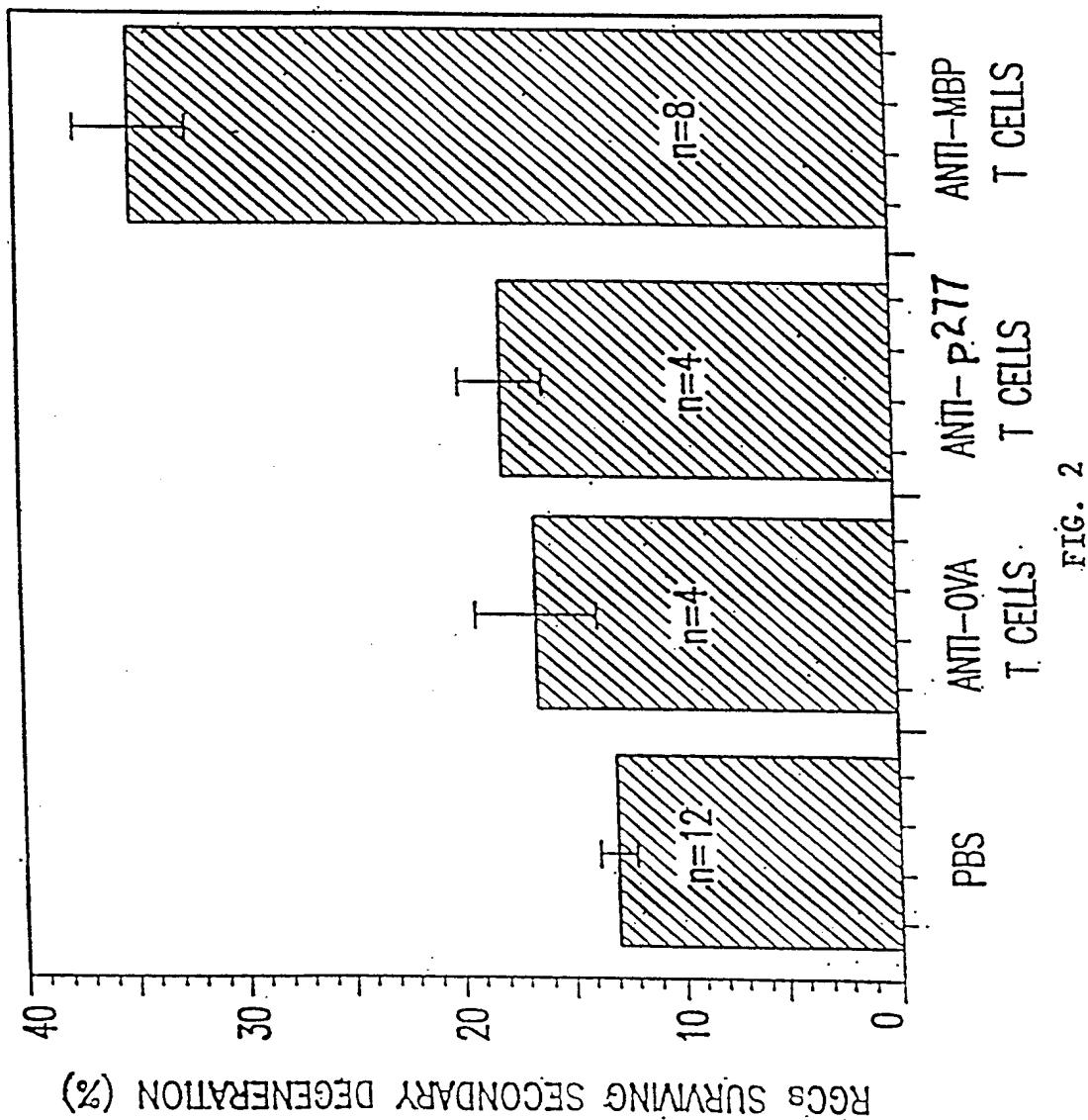


FIG. 2

160 μ m

FIG. 3C

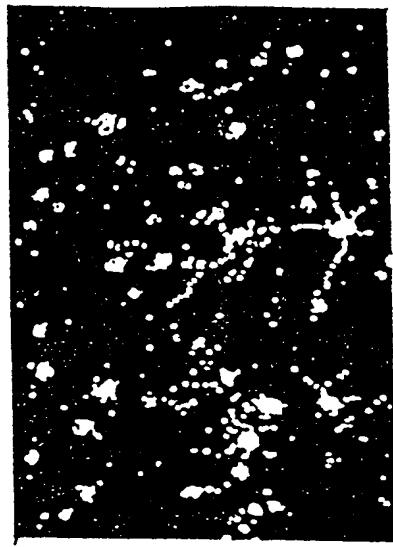


FIG. 3B



FIG. 3A



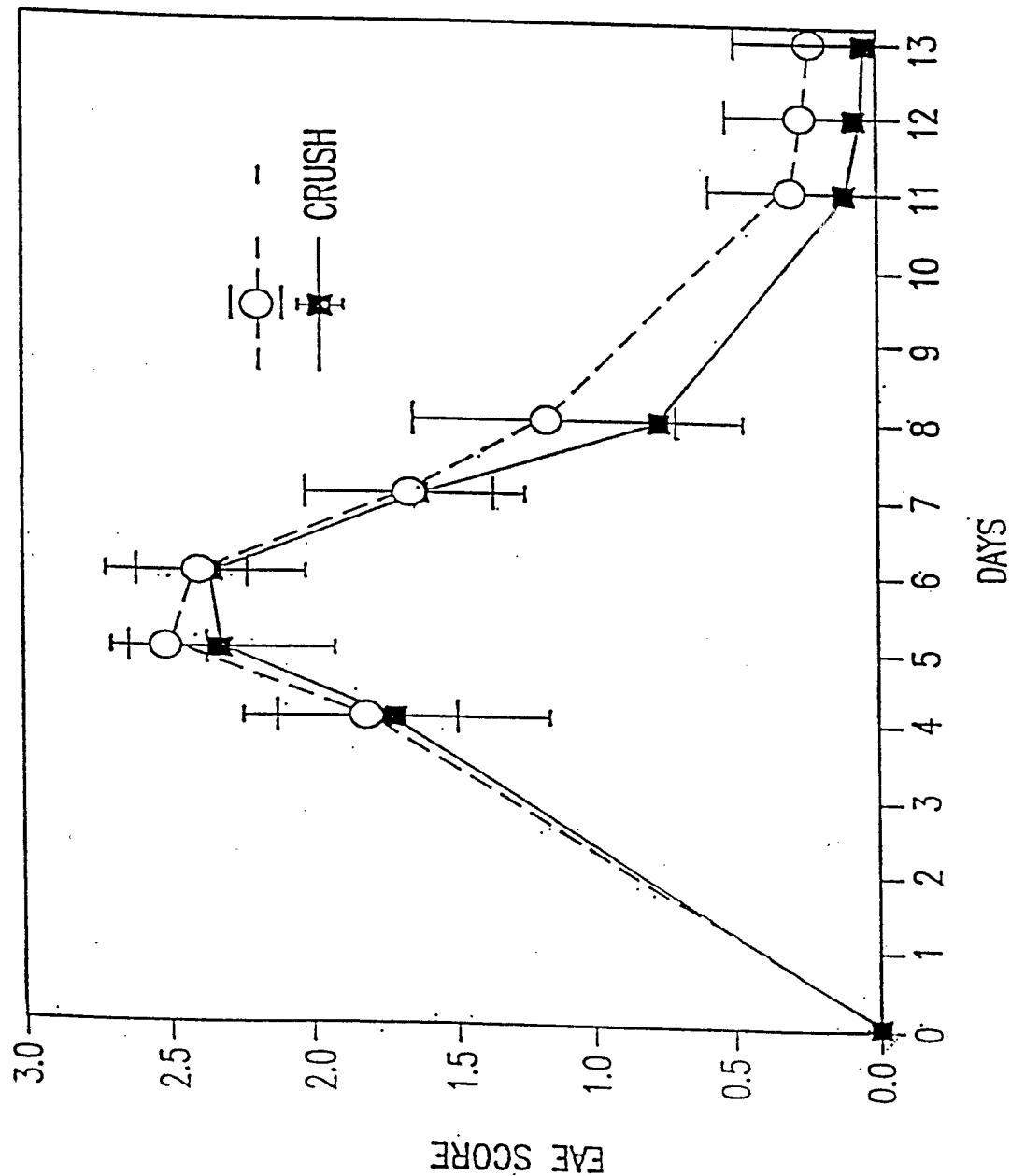


FIG. 4A

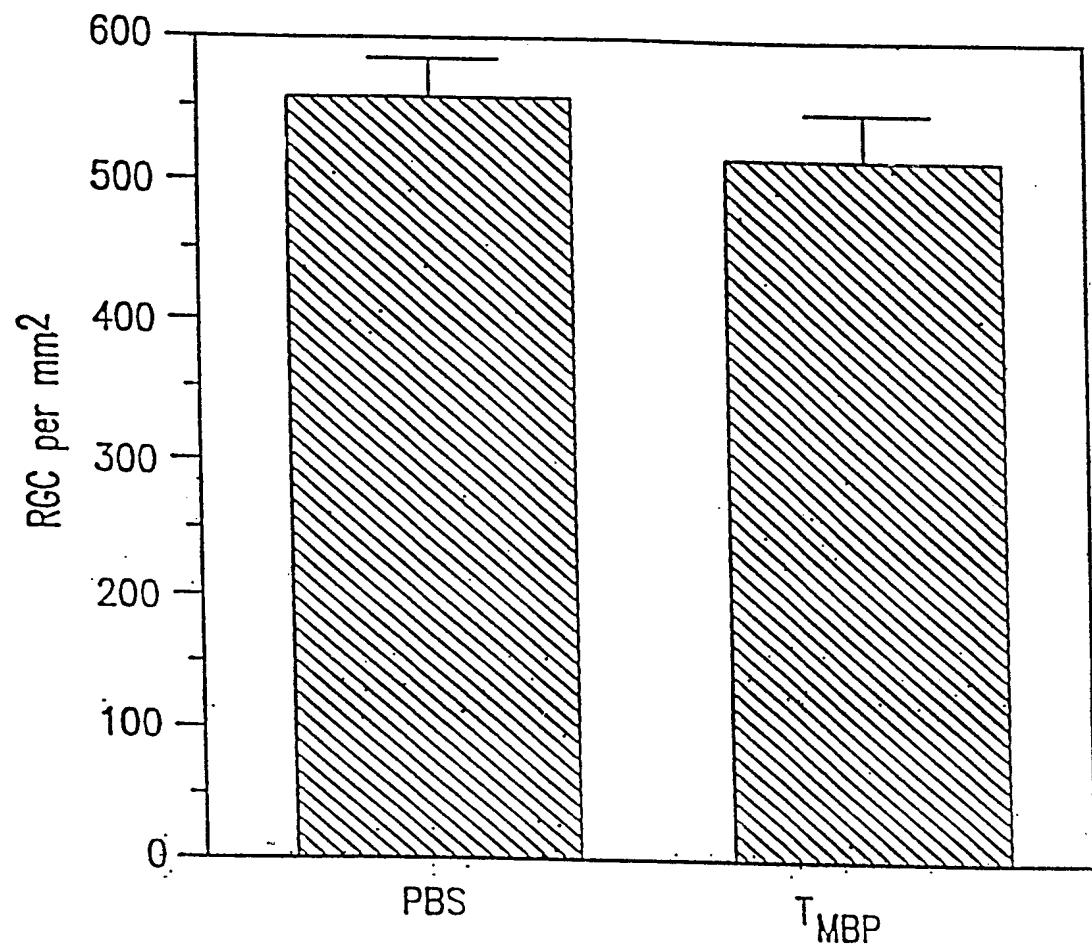


FIG. 4B

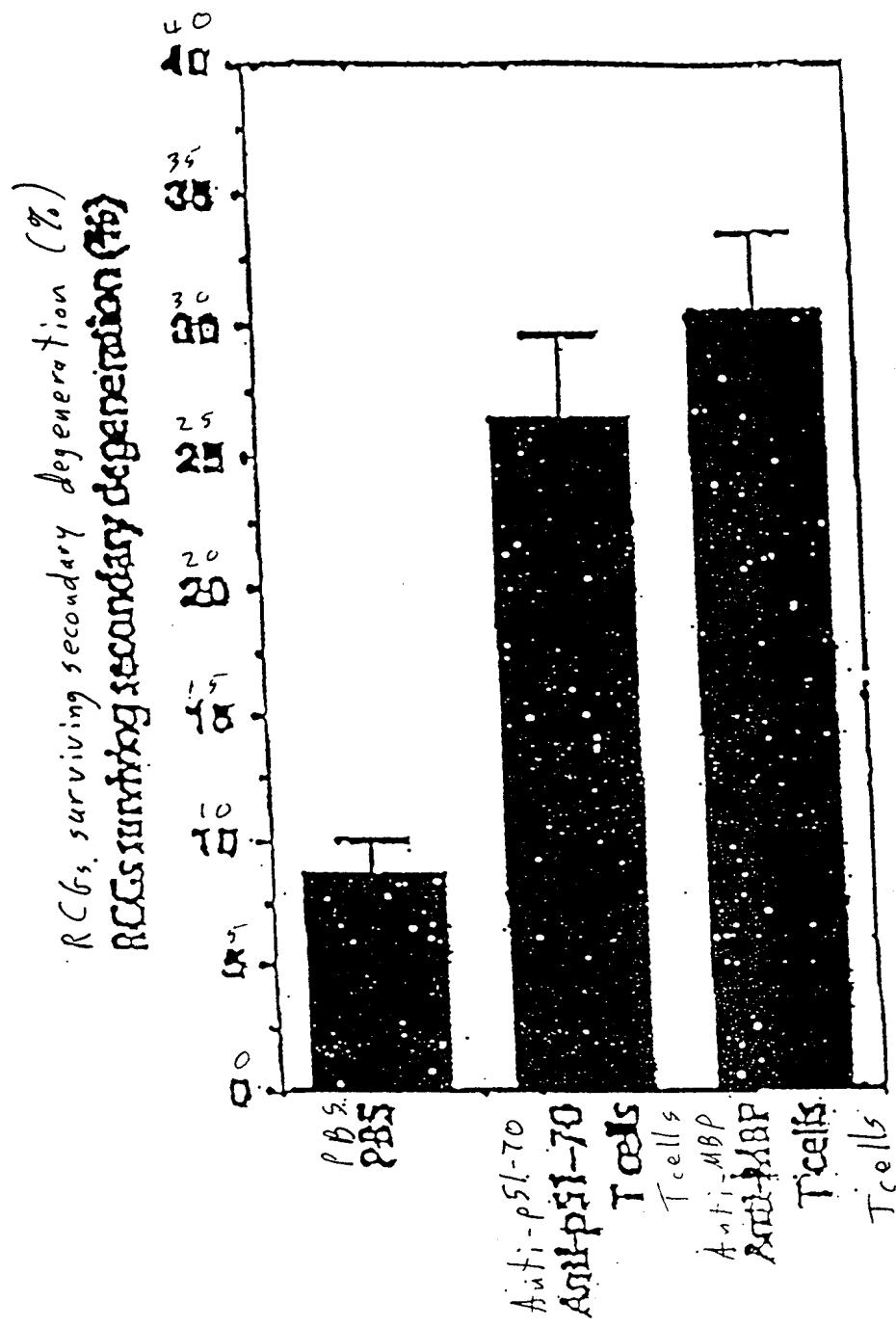


FIG. 5

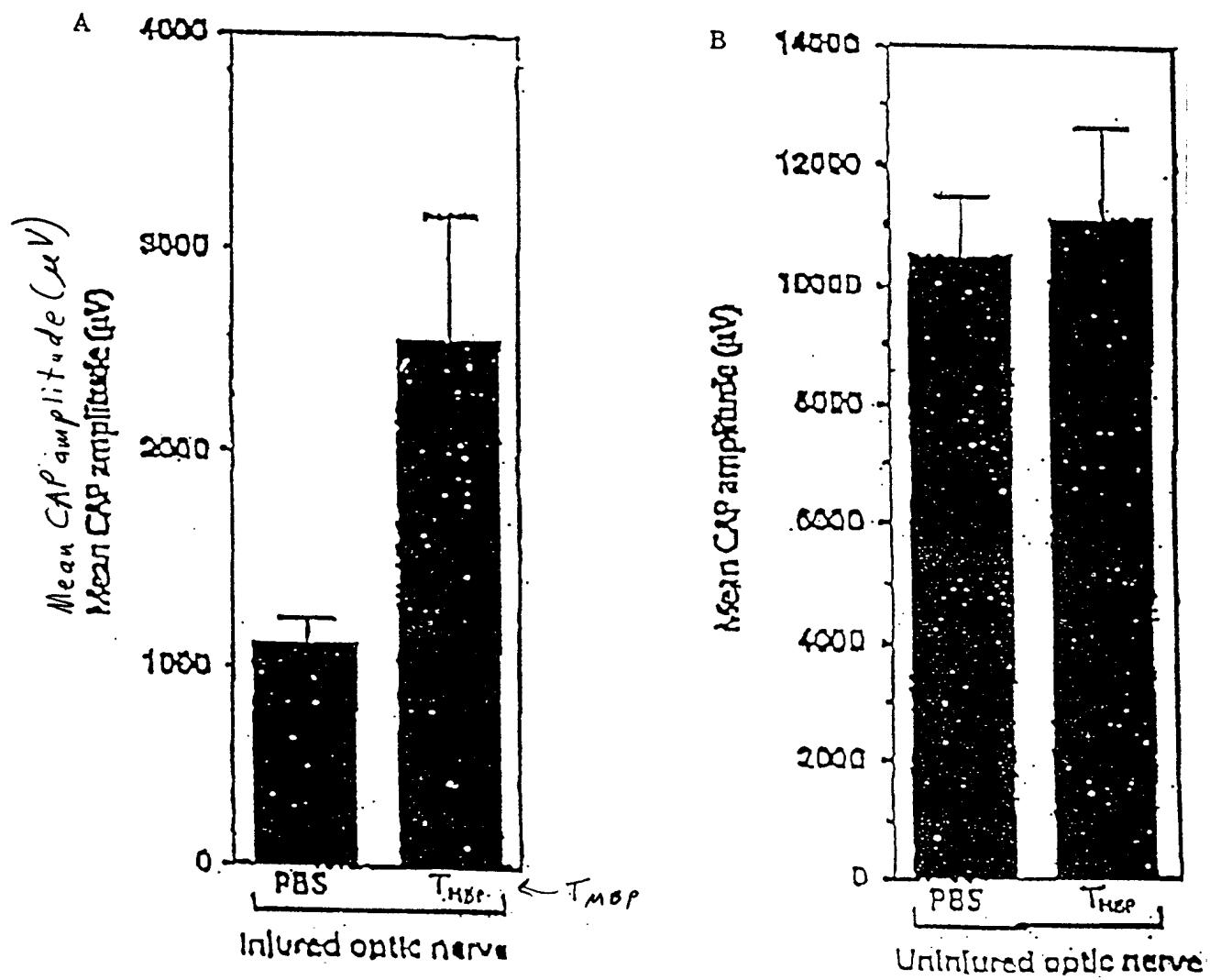


FIG. 6

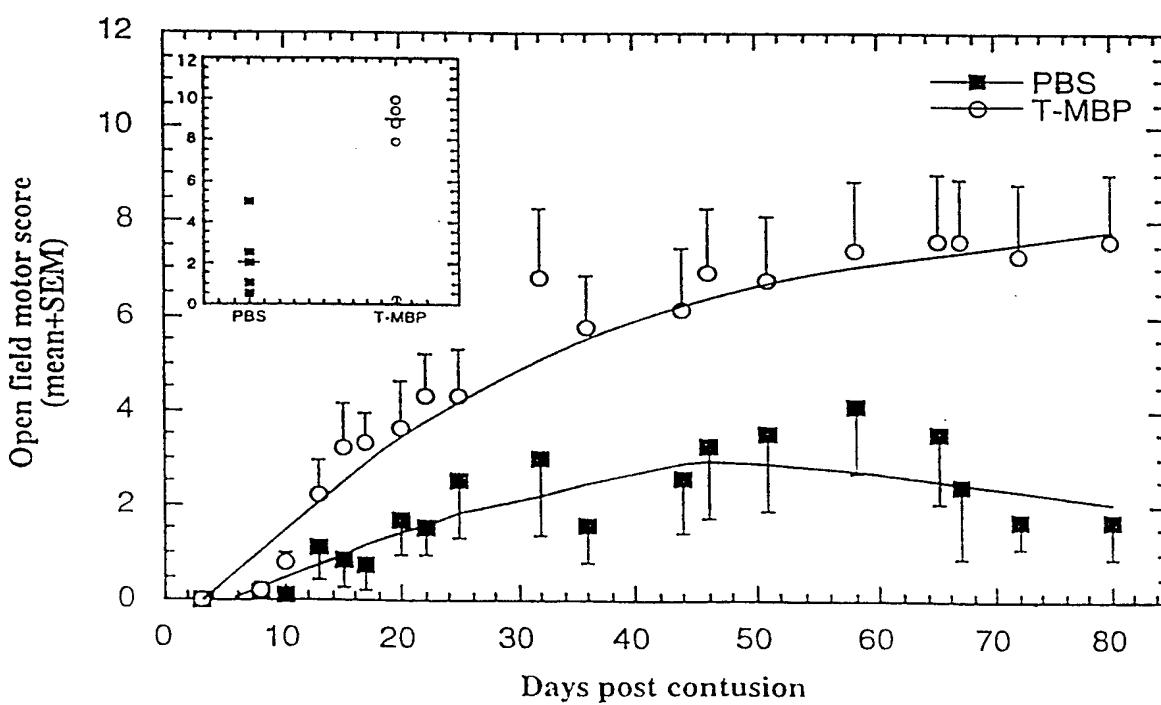
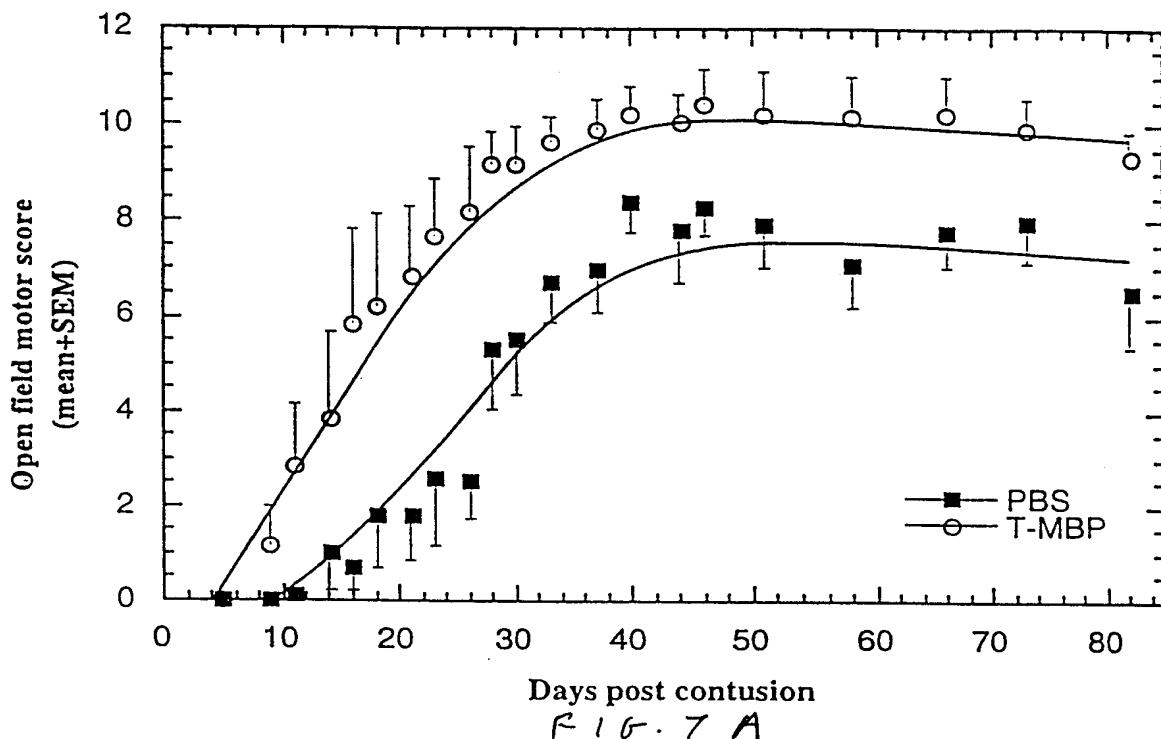


FIG. 8

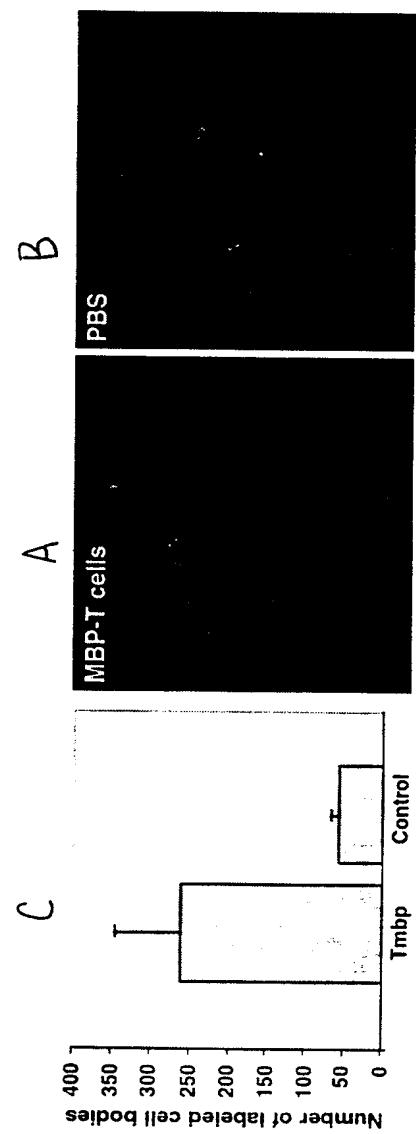
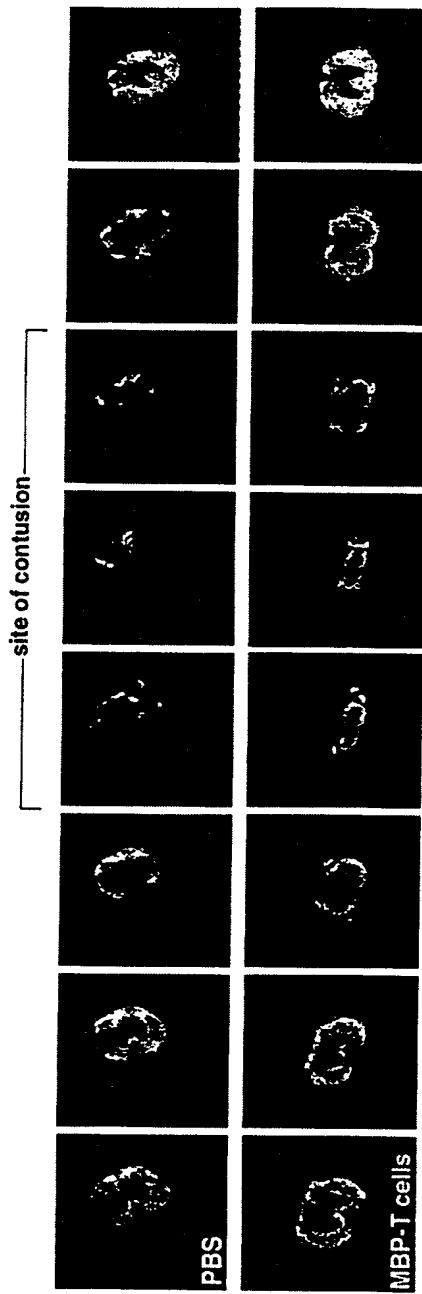


FIG. 9



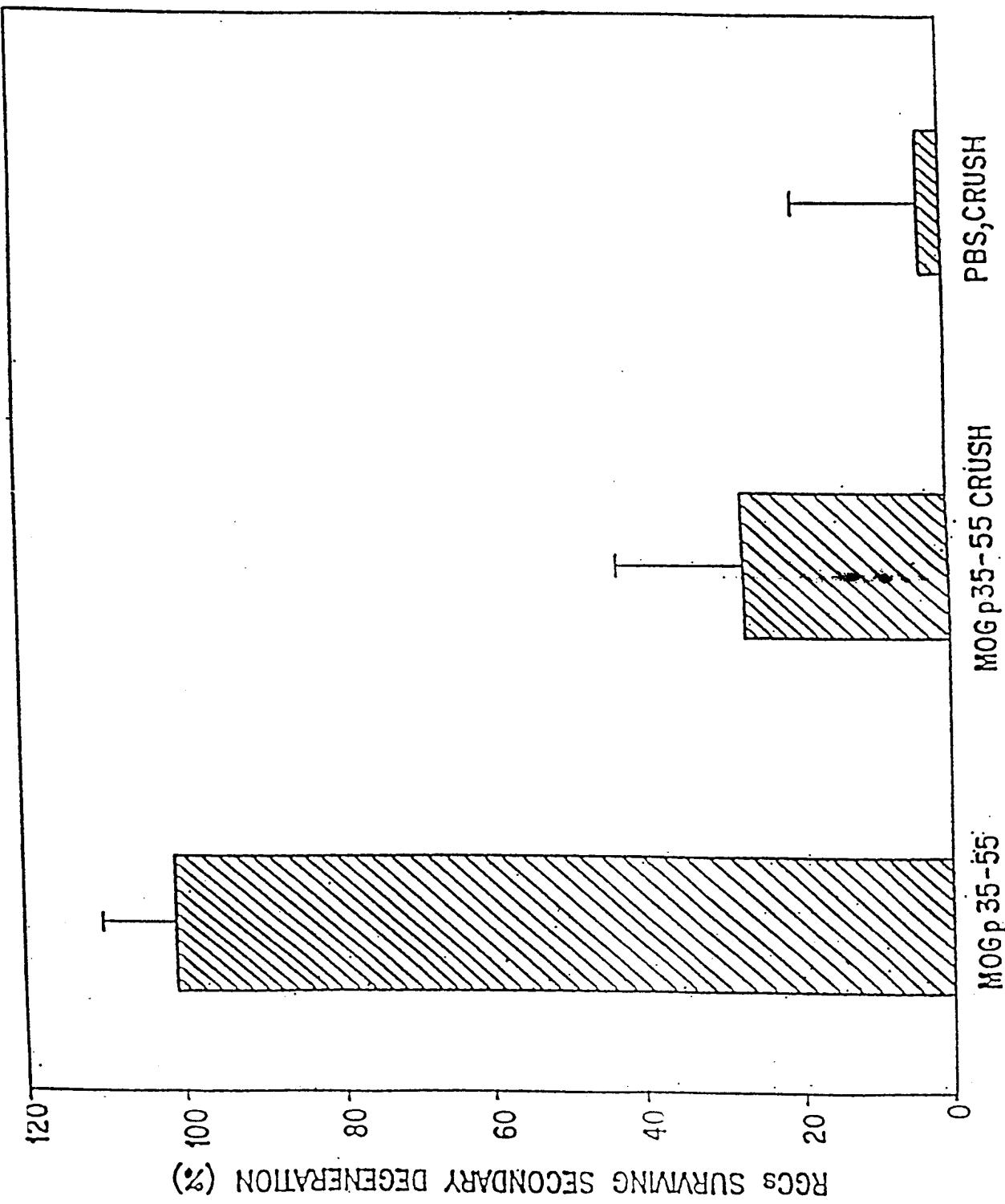


FIG. 10

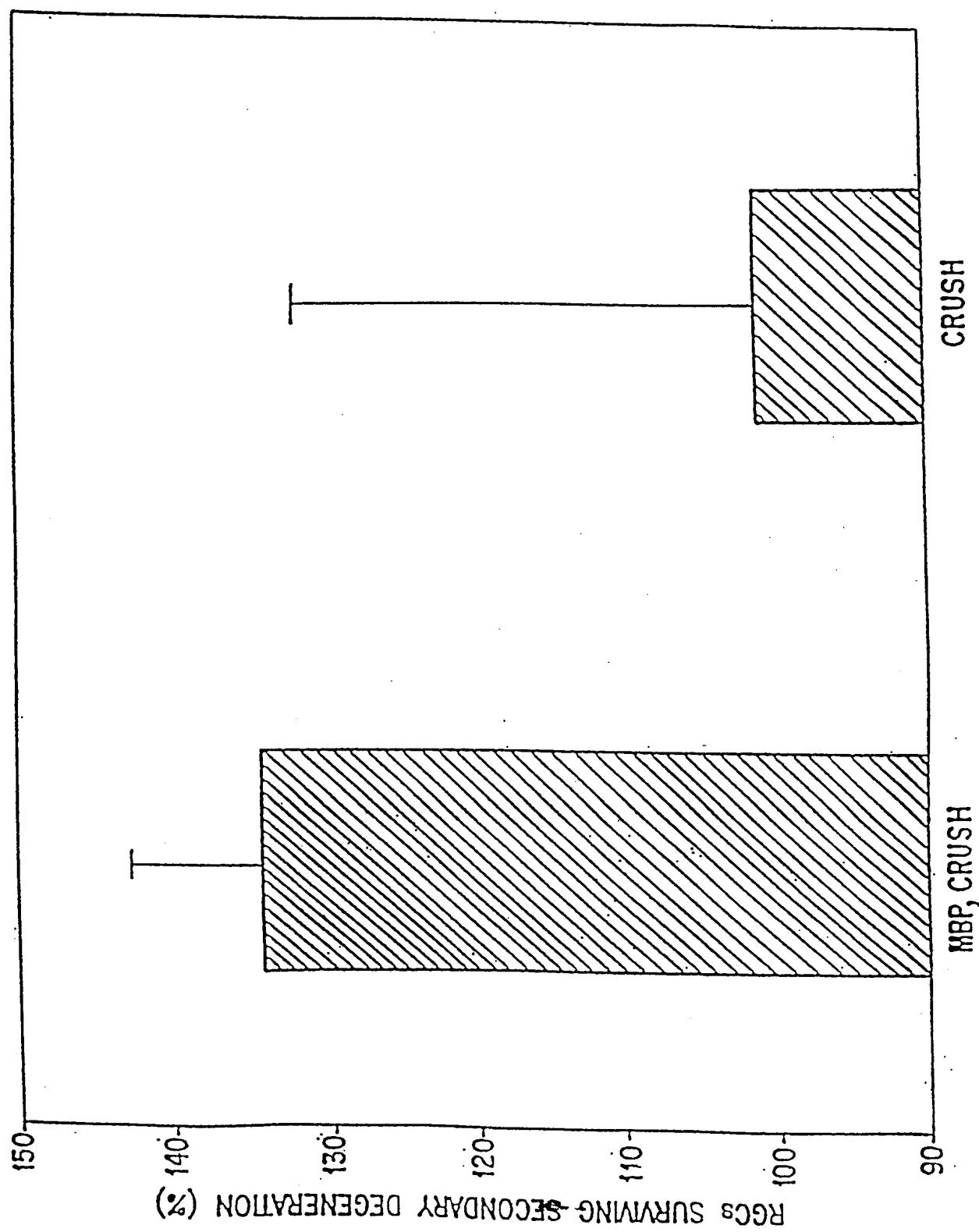


FIG.12

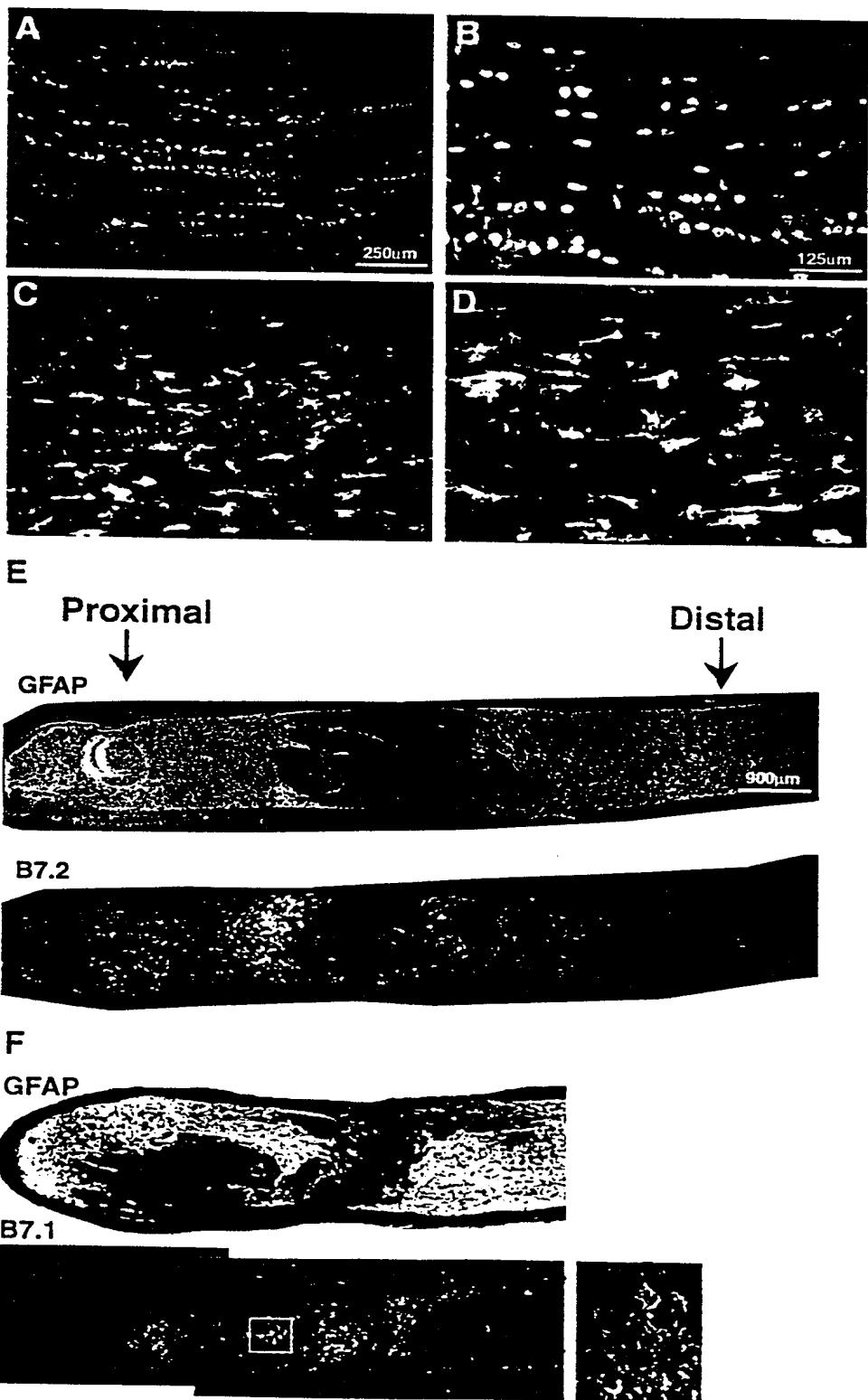


FIG. 13

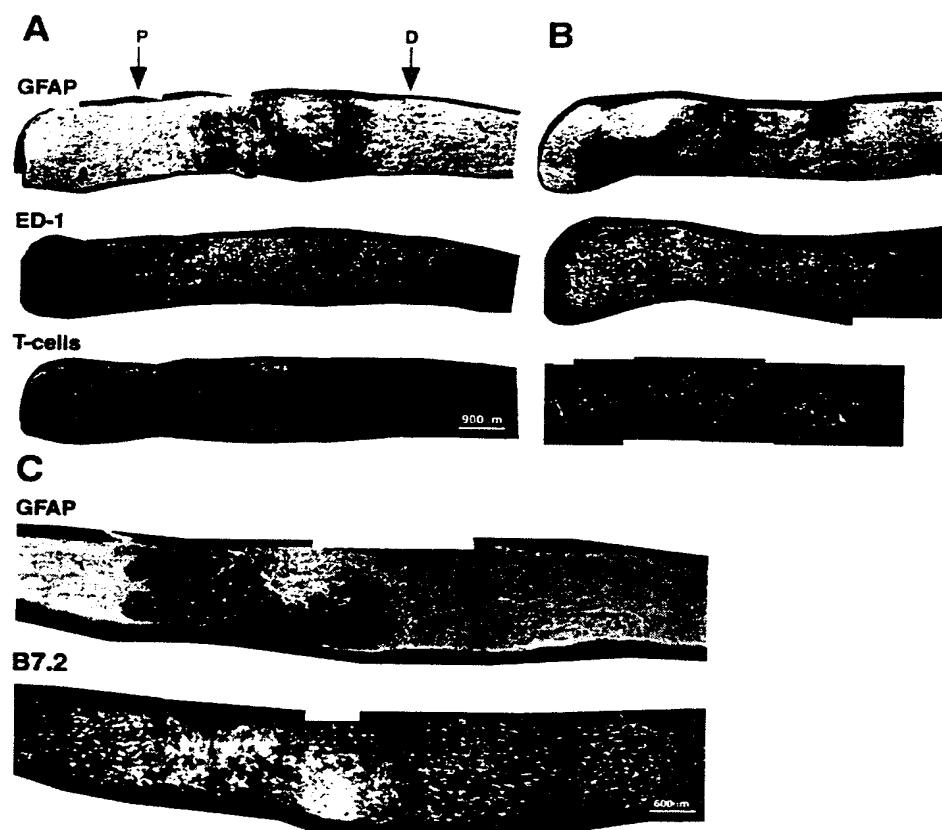
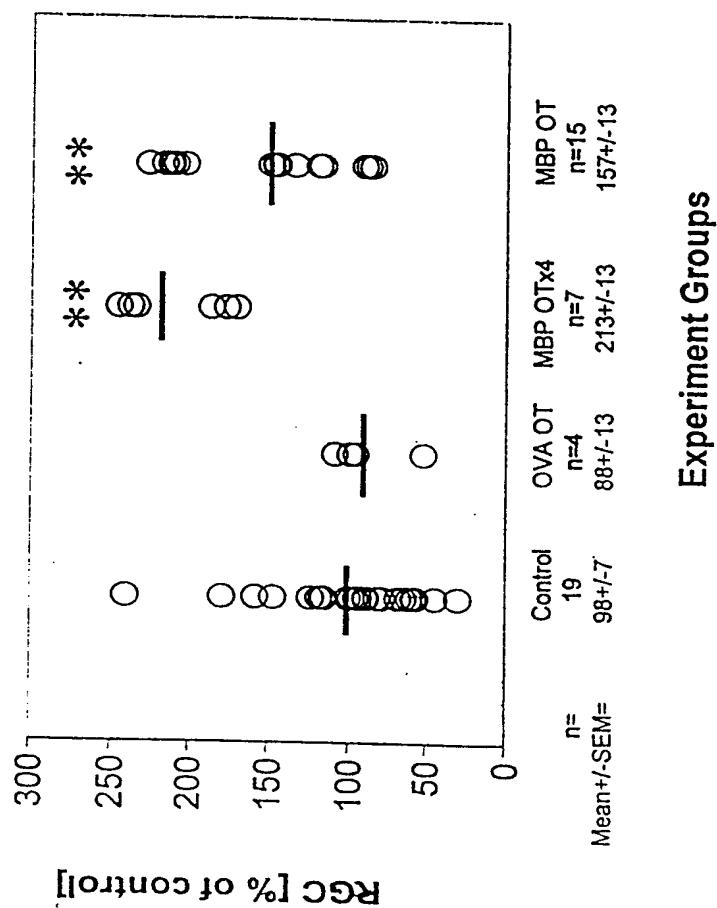


FIG. 14



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541 cccgtggggc tgtgaacgcg gcgggcccagc ccacggcacc ctgactggct aaaactgttt
601 gtccctttt at

FIG. 15

1 gaaaacagtg cagccacacc 61 cagaggcactg gatccaagta 121 ttcctcccaa ggcacagaga 181 gacaggggtg cgccaaagcg 241 cactatggct ccctgcccc 301 cacttcttca agaacattgt 361 ggactgtccc tgagcagatt 421 ggaggcagag cgtccgacta 481 ggcacgctt cccaaatttt 541 gctagacgct gaaaaccac 601 ccttaaaact ttaatccac 661 aatgcctgag gagttgtca 721 ggcacacagt taaaatgagaa 781 gaagatcacg ttccctcccc 841 aggtgcacag acgtccctcc 901 ctcggcaccc ttgcgtttt 961 gaggacgtgg gcggcagaga 1021 tccttctctc acctcctaaa 1081 gtggcttact acatgtggga 1141 gtccaaacgtg gggcagcada 1201 cccaaattagc tgaattcgcg 1261 aatggcctca cataggaac 1321 gtggcctctg gacgtccacgg 1381 cctcgtttcc aaaccacago 1441 agaagggttag gagtcctcta 1501 tgtgaccggg cgtgctggca 1561 gataaccggc tcattcactt 1621 cacctgtctcc cgaattactc 1681 gacaaaagacg tcttctgacc 1741 ccctccctaa cctactgacc 1801 caaagagttcc cacgctggca 1861 atgccaaaac gcgtcttctt 1921 ccatgtctat taatatatacg 1981 acaaaccctcc aaatttttca 2041 gatccatacg tggccgcacc 2101 acactgttcc tgaatattga 61 cctggccaca gcaagtacca 121 cacgggcatac cttgactcca 181 gggctctggc aaggactcac 241 gaagtacacac ggccggaccc 301 gacgcctcgc acaccacccc 361 tagctggggg gccgaaggcc 421 taaatcggtc cacaaggat 481 taagctggga ggaagagata 541 ctgggtccgg aatcctgtcc 601 ttgccccctgt tacctaatta 661 cgttagtaggg tcagggccacg 721 catgaaaaca gaaaacgggt 781 cgcaatgtgc ccccagacgc 841 acgttcaccc ctccacccctt 901 gctggtcaact gccatggagg 961 ggactgttga catccaagct 1021 gttagacttca tttttcctaa 1081 gcttttgggt atgtgacatg 1141 gagagggggc cacctcccc 1201 tgtggcagag ggaggaaaag 1261 agggtcttcc tggagatttg 1321 ttgccctgca tggggccccc 1381 ccacagccgg agagtcacgg 1441 gacagctcg cagccgcgc 1501 gggcagtgcc acagtggcca 1561 tcattcactt cctcccaagaa 1621 accggagacac acgggctgag 1681 atatcttct taacacccgc 1741 cacctttga ttttagcgca 1801 tcaccctccc cgaggacgga 1861 aatccaattc taattctgaa 1921 cctcgatgtat gagagagtt 1981 gcagaagcac tctgcgtcgc 2041 cacacagcac gtgctgtac 2101 aataaaacaa aataactttt

FIG. 16.

A.

```

1 taatatctag ggktttgact ctgaccgcgtg ttggggctct cacttcatgg ctttcacgc
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121 ttcattttat ttccacccctc aatccacatt tccagatgtc tctgcagcaa agcgaattc
181 caggcaagcc ttagggaaaa aagaaaaaac aaagaaaatg aaacaattgg cagtgaaagg
241 cagaaagaga agatggagcc cttagagaag ggagtatccc tgtagtaggtg gggaaaagg
301 gaggagaagg ggaggaggag aggaggagga aagcaggcct gtccctttaa gggggttggc
361 tgtcaatcag aaagccctt tcattgcagg agaagaggac aaagatactc agagagaaaa
421 agtaaaagac cgaagaagga ggctggagag accaggatcc ttccagctga acaaagtca
481 ccacaaagca gactagccag ccgctacaa ttggagtca gatcccaa acatggtaa
541 gtttcaaaaa ctttagcatt gaagattcaa gaggacacag g

```

B.

```

1 ctgcttcag agcctgtgac ttcttgtgtg cctctccgt ttctcagcaa catggcatag
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241 tagccaaagct gcattataagg aggacaaaac tgccatataaa agtgtccaaa atcattaagc
301 ctgcattttt attattggaa gtaatataaa acctccattt tccaattttt catttcttgc
361 cctgtgctag ctccatcctg tttggactgc tcccccata taaaactaa gaagaatcaa
421 gcatttttg caacaaatac acacgatgct caaaaatgtc caggagcatc caatttccaa
481 agtttctcc accttggaaatg ctcttcatgc taaaatcctg tctgacaata ccagcatctc
541 tggcctgcac tcatcccttc ctgaaactcc aagtgcattt accctctgtt accacttaat
601 tggctgcctg aattgttagt tgaaaatatt aggtctactt agctaatttt tcctcaggaa
661 attaaagact cccatatggc agagtctgtg tctttctct cttcatatcc cgtataacac
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781 agcaagcaaa caaacagact agaaacaataa gaaagaagg actggatttc ataatcttc
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961 ctcttactgc ccaggctggta gtacaatggc gcaatctgg ctcacggcaa cctccgcctc
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1621 gtgttaagtac ctgcctccccc acacagaccc atctttttt tccctctctc catcctggag
1681 atagagaact cttcagtagacc ttagtaacta gcaggggact ggggtggagc cagaccggat
1741 tcccgagtc tccctctgtg ca

```

FIGS. A-B

C.	1 ctagaaaatc cctagccctg ttaagggtct cgctctgggtataacctcac ttatgtcgaa 61 aaagaagcca ggtcttcattataaggatt ccctgggtctc gtttgcctac ctgttaatgc 121 aggatccatg cttccagta tgtcatctat ggaactgcct ctttcttctt ctttatggc 181 gcccctcctgc tggctgaggg cttctacacc accggcgca tcaggcagat ctttggcgac 241 tacaagacca ccatctgcgg caagggcctg akgcaacgg taacaggggg ccagaagggg 301 aggggttcca gaggccaaca tcaagctcat tcttggagc gggtgttca ttgtttggca 361 aaatggctag gacatcccga caaggtgatc atccctagga ttttgttca ataacaagggg 421 gtgggggaaa attggcgcg agtctgttgc ctcgtccccca cccaaaggctg gtcctctt 481 aggggcctgg catttgagtg aggaagcgat ggctgcagcc gaacgagaag gtcaggaaaga 541 acgtggtgcc cagctggctt agcctcacct ttcaaagggtt ccctaagcaa atttcttctc 601 aaaacagaaaa gcatgagttt tggggatgc tttgtacaat cagaccattt ctaagccatc 661 tgggtgtatc ctttgttcc cttctagta ggtaccacaa ggttggatct aactggacaa 721 gagtctaaaaa tgctgctcat gtgattgaga cttgggcacc tgagctrage gggaggatgg 781 ataataaaaaa ttaataata actccaaatg aaatttacaa tgttctgg
D.	1 gatcctcctc attcttcccc tacccattcc ccccacccctc cgttatactg gggccaggta 61 tcttagtagat actgccaatt acccttgca gaggtgcct gctactaat ttatgttgg 121 ggagmgccct ggaacctgg tttatgtct ggcacacgccc acttccagga tctcccaatt 181 tgggtttcta catctgcagg ctgatgtca tttctaaccat acccatgtca atcattttag 241 tttgtgggca tcacccatgc cctgaccgtt gtgtggctcc tgggtttgc ctgctctgt 301 gtgcctgtgt acatttactt caaacacctgg accacactgccc agtctattgc ctcccccaagc 361 aagacactcg ccagttatagg cagttctgt gctgatgcca gaatgtatgg tgagtttaggg 421 tacgggtgtt ttggctctcc tacccactat ggaagcacta tatattttggg tattttctta 481 gtgttaaggaa ggtgtgtattt atgagaaaaaaa tataagatga tgaatgattt ggttttagtt 541 tataatccct tccctactga aaccagagag gtttcttccc cccggaaaggga acttggaaagt 601 ggtgggagtt ttcttggcca ttcacatgg cctactctag ttgactgtcg ttccacaaccc 661 caaagcagca catttcaata acaaacacaa gttttsacca ctgttcaata ccaccccttc 721 tttttgttaa acctgttagaa aagaggatcc taattgttgg tagmatccaa mtttacagcc 781 aggataatta gagatggaaag aagggtctcg gggggaaagtc tccatgtggc cccgtaaact 841 cataaaagctt accctgttgc cttttgtgt ctactttagg ttttcttccc tggaaatgttt 901 tccctggcaa ggtttgtggc tccaaacctc tggccatctg caaaacagct gaggtgagtt 961 ggttattttgg gtttattttac aagggtgtt gtaataccat acaaattaca cccatggctt 1021 tcaattttaa ggactgaaag ttcccttgc ctggattttgc aatttagccgaa ttgccttctt 1081 caacatgttgc gctaaatgttgc cctgagccaa tgagcataga aggtaaaaaca cctttttctt
E.	1 aatttagcaca cagaaaggat atccaaacaca tacaaggctg tnntcatggc ctacactggaa 61 gcatattact gctgttgcac gaaacatttc ttcttcttct tttcattttc ctgcagttcc 121 aaatgacctt ccacccgttt attgctgtcat ttgtgggggc tgcagctaca ctggtttcccc 181 tggtgagttt accttgaatg atcttggccaa gtaaatagggc ctgagatagt tgggttacaa 241 gctatctga aaggcaagaa ggttagactgc ttccatccctt gaaatgttgg aggaa

FIGS. 17C-E

F.

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421	gacatatt	tgtggcaa	cccaaat	gttca	ctgagg	actcg
481	gatcaa	aggtgtgg	taatctt	tgattt	ctctgg	ggccat
541	accatgat	cctca	atcggact	cagtca	tatatgg	aagcca
601	tggatgt	tgctttt	agagcat	ttttcc	attttagt	gattt
661	ctacc	tcattcc	agggatt	ggagg	cttctt	tactct
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781	ccgtcctt	actcatgg	cgagg	agttctg	ccccgt	atcccc
841	ctcta	gaggctct	ccacac	tacaatgt	cgtctcc	cttaact
901	tgcc	accaactgg	ccttctt	cttgat	gtaaca	aggag
961	tgc	aagg	tttggact	ccctctt	gtac	ttttagt
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1081	aagtca	ggaatgg	ctcta	atttca	atctcc	gatcagaa
1141	taatttctt	tcaaagg	cttcc	tggaaaca	gtgg	aggaa
1201	gtc	aaatgtt	tggctctt	ccatctt	ggggcc	aatat
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1501	gacgaa	ggg	gcatttgg	ttacac	aaacagg	ttgtcag
1561	tcttc	act	cccttctt	tgataac	accctgt	ttccaagg
1621	ctgaga	atag	aaggaaact	gcttacat	gcctgagg	cagcag
1681	tgg	ctaa	tggtaacc	tgagatgg	cacagg	ataactt
1741	ggat	agcat	tctttttt	tgttaatt	tggctct	cataatct
1801	caat	ggtg	catttcat	gttattat	attcagt	cgtagg
1861	tgat	ttt	tagaatgt	cacattt	gtattcc	ttgttatt
1921	gttgc	atc	aaatgtct	agaataatt	tttgattat	actgtt
1981	aatt	ggacat	taagcat	aatgatatt	aaaaatt	aaacttag
2041	ttc	taca	attctgc	tgcagaa	ttgtgat	tattgg
2101	aat	ctgt	ttcttct	gttattat	caattt	caattt
2161	tgtt	aaag	taggtt	ataatgg	tttagca	tatctt
2221	tgca	cagg	atcag	tttctct	atata	cataacag
2281	gttccc	cagg	aaaagg	cagttgg	gttccag	cagtgg
2341	gtcttta	at	ttttgtt	ggcagaa	tttgagac	ccagatt
2401	tgaagg	aaag	atttgtact	acttgg	tgaatag	tggttta
2461	caaagt	gggt	tca	catttt	ttgtgtt	tcttttcc
2521	ttgtt	tc	accgtata	ttaaagg	gttgaaga	tacaaa
2581	atgtt	ttgt	agaaacca	aagataa	taatccgt	cacttgata
2641	gttt	gatt	aataacata	caagataa	gacagtga	tttggtt
2701	tacacat	ata	tacacacaca	caaataac	acccttcc	catgcata
2761	atagaatt	gtc	aatttta	aagagag	aaaacct	catttctgt
2821	gtat	tttata	aaattaa	ataaaactt	ataaactt	tttaca
2881	ttgag	gt	tgttctt	gacttca	tagaaactt	ataatcg

FIG. 17 F

1 atggaaatgt tctgtatttg tgggtctga tggataacc actaactgta gtgctatttg
61 gcatttggaa catggctagt gtaatcaatg aaccaaattt ttaattttat ttaatttgtaa
121 ttaattttaa gtggccacat gcagggagtg actgctgcat tggacagcac ggctctaaat
181 tgagccttt ttccttattt ggtgaggcat acttgcctta agatggaa gtctatttt
241 gaaacctgct accaatgctg gtctcacact tgcaattctc agctgagcca agaggtgaga
301 gaaaggctat ttccattcc aagatctac tctccctgt gacactgagg aaactggcaa
361 gtgatgtgaa ggctggagag cgtgtctgt atgctggctc tgccttctc gcctgtttg
421 actgacatag ttagttgctg ccctgctgg tcccttccc tccaaccttgc cctctctgag
481 cacacctgac attcatctca tgacttccct aaaaacattc tttgggaaca agaaaactaac
541 aaatcccaag tgaccttatca catataaaaa catacagggc agagtttggaa ttcgcggtag
601 aagaaaggga ggttagacat taagaagaat ggtctggta tgacagttgt gagataatag
661 aaacaggaaa aaaaatcta agtttctt cttttttaa gaaccaataa taatttctct
721 ctttgacta gtcagtaggg ctgggttga ttggaggaag cttacatatt ccatgaacaa
781 gcctcttctt aaggctctgt aagtgtatcct gccccactga ttagcccccta gaagaccctt
841 caaagggttg atctccagga gggagtgggg gaggaaagcc ctgtaccagg cagcctctgc
901 tccattgtct tgggggggtg gggaaagacaa accctggtca tcccctcagt ctgtagccct
961 tttgtgtgag tgcctggcaa gggtaacgtg gggctgtttc tgcgggcaca gctgcagcaa
1021 taccggagt ggagggcaggg cccaggcage actgccttcc aagatcttcc cttgggctt
1081 tcagcagtaa gggacatgc accccaaggg cctccacttg gcctgaccctt gctgcggggg
1141 ctctctgtc ccaggaacag tagagatggc aagcttatecg agaccctctc tgcggcagctg
1201 cctctgtctc ttctcttcc teetcttcc ccaagtgtct tccagctatg caggtaaagac
1261 .atgtttttt tcctgcctg gggagaccct gaaaacagaaa aggtagttt cctgggggtt
1321 agctccttca aacatcctca 1381 tgcctccctt cctcagaaaac gttccgtggg ttagcttcc acactgtaca
1381 tgcctccctt cctcagaaaac 1441 gactctttt ttttttggg acagagtctc cctctgttg ctaggctgga
1501 atgatctcgg ctcaactgcaa 1561 tcccaagtag cgagactac 1621 ttctttttt tttttttgg
1681 gtctcgatct ctgacactgc 1741 ggcgtgagcc actgcactag 1801 tggtgccag gctggctgg
1861 aagttctgag attacaggca 1921 ctccaggcct tccttggact 1981 caattgagat aaaataataa
2041 tatgtAACAT gcttagcagt 2101 taatatcttgc tcatgtttga 2161 aaggAACCCG tctgtcacgt
2221 gccacactgac tgacagagggc 2281 ttatcttca gtagctcaga 2341 aacttttcg ggtcagggtc
2401 gggaaataga gccatcccc 2461 gaacaaggac tgaatagtga 2521 accaaaaacct ctaagggtag
2581 taggaggatt caacactcaag 2641 tgaagagtc gatggctca 2701 tgctaaaatg ttctgtccag
2761 ttgttgttct caagaaggaa 2821 cgcttgggg ccaaggcagg 2881 acagtgtgaa acctcatctt
2941 cctgtaatgc cagctactcg 3001 aggttgcagg gaggcggagg

FIG. 18

3061 ggcacagaga gtaagactgt ctcaaaaaat aaatgaataa ataaaaagga agaagaagaa
 3121 gaagaacaat tgcaatcctc cctggctcta gaatgtcatt taaaagtgcg gtgtcttctt
 3181 ccttcctgt ttgtggcg cccttctcat gacaggctt gttggccaagg ttccctctga
 3241 ccttaatct cttccctttt ggttggcg cagggcagtt cagagtata gaccaagac
 3301 acctatccg ggctctggc ggggatgaag tggatttgc atgtcgata tctcctggg
 3361 agaacgtac aggcattggg gttgggggtt accggccccccc cttctctagg gtggttcatc
 3421 tctacagaaa tggcaaggac caagatggg accaggcacc tgaatatcg gccggacag
 3481 agctgctgaa agatgttatt ggtggggaa aggtgactt caggatccgg aatgttaaggt
 3541 tctcagatga aggaggttt acctgttct tccagatca ttcttaccaa gaggaggcag
 3601 caatggatt gaaatggaa ggtggatgtt gccatataat attgttatt aactgttggg
 3661 tggcaagaa caattattct ctcaacttgat atgagatccc tcaacccaaa catctcagtc
 3721 ctggaaatga tttccataaa aatgtacaca tcaataaaca gaaactcatg ctttagggatg
 3781 tctgttgcatttattcag agtagcaagg aaattggat caaaatcaat gcctttgagtt
 3841 aggttaatgtgaa cagaatgaac aatggtagcc atactgtgaa tattatgcag gattaaaaaa
 3901 gattatTTTA gcactaggcc agatggttt gggggctcct ctaaggtatt attgagtgat
 3961 aagagcaagc tgctgttaga tacaaaaaca aaaacaaaaac cctagggcat gttggttgc
 4021 ctcgcagcta ctcaggaggc tgagacggg ggttggctt agcccaagggg tttgcagta
 4081 cagtgcgatc tgattgcacc actgcactcc aacccgggtt acagagcaaa gacccatcacc
 4141 cccactccct acccgctctt aaaaaaaaaa aaaaaaaaaa caaaaaaaaacc cttgggcca
 4201 gcccgtggc tcacgcctgt aatcccagca ctgtgggagg ccgagggtggg cagatcacaa
 4261 ggtcaggaga tcgagaccat cttggctaaa acgtgaaac cccgtctcta ctaaaaatac
 4321 aaaaaaaaaa aaaaaattta gcccaggcatg ttagcaggcg cctgtatgcc cagctactcg
 4381 ggaggctgag gcaggagaat ggcgtgaaacc cgaaagcggg gttgcagtg agccaaaatc
 4441 ctccactgc actccagcat gggggacaca gcgagactcc gtcctaaaaa aaaaaaaaaa
 4501 accctgtatt tgtgagcgc cacacacaca cacacacaca cacacctgtt cttggtccta
 4561 gtgataaagc aagtaatca aatgtctaaa tataattata gaaaggagat gtcaccttt
 4621 ggctgtactt ccactatttcc attctgcaga attgcagaat ttctttttt tttccttct
 4681 ttcttttctt tttttttttgc acacagatc tcgtctgtt aaccaggctg ggtgcataatg
 4741 gcccctccg ctcctgggt tcaagtgtt ctccctgc tggccccc gtagctggg
 4801 ttacaggtgc ccaccaccac acccagctaa tttttgtatt tttagtagag acagggtttc
 4861 accaggttgt caaggttgtt ctcaactcc tgacctcagg tgatccactc gcctcagact
 4921 cccaaagtgc tgggattaca ggcattggcc atgggtcccc gctcagaat ttcatttca
 4981 acatgttttgc catgtgggt gatttggag aatattttt gctctatcgc aggttatttgc
 5041 agatgtggac aaggtgaagc cgatggaggg ggagcttga aagttacttgc tattttattt
 5101 gagaactaa actgttttgc gacccctgggg gtcagatccct ctgcctttc ctccctcccc
 5161 cctgcagtgc aaacatcaga caattgtatc ctattgtatc ttggaggtgg ggtgaccat
 5221 tgcagtgcgtt ggaccagaag atggcatgtt atgtggaaaca acaaaagact atttcttagag
 5281 actgcctgcg gggatatggaa aatagcttta tgggtctcgtt aatgttcttc atacagctgt
 5341 ttttatttttttgg gaaattctac ttggcgaaaa gtttggatgtt gagaccctt ccagttgc
 5401 gatTTTCTC CTTCCGTCTC aacaacttcc tagctcagta actgccttc ccaacaaact
 5461 ccctcagttt caccacacca aaaaaggaaag acaagccggc tgcggggct cacacctata
 5521 atccccaaaac tttggggaggc cgaggccgggt ggatccaccc gaggtcggg gttcgagact
 5581 agcctgacca acatggagaa accctgtctc tactaaaaac acaaaattag cctggcgtgg
 5641 tggcgcatcc ctgtatcc agctggggagg ctgaggccgg agaatcgctt gaaacccgg
 5701 ggccggagggtt gcaatggcc agatcggtt catatcactc cagtcggc aagaaaagtg
 5761 gaactccatc tccaaaaaaa aaaaaaaaaa aacaaggaaag acaaaaaagaa aagcagctaa
 5821 agacttttgc tcaaggggaga aagttctt tgggttgc atccacattc caaccccttgc
 5881 ttccacccctc ttgtgtgc tgcctaaagaa actgttttac aagttatataa gggacgctt
 5941 gtcttaggtt tggagccagg aagttggagac aatatttagga atggatgaa gtaatggat
 6001 tatttgcattt ctcagggtt actacccctt cttttcttca aagagtttcaatccct
 6061 ttttttctt ttttttttttgcatttttggattttatttactgttgc tctaattccct

FIG. 18 (cont.)

6121 tctttaaatt cttcattatg aaacataaaa acaaatgcc a ggcgcggcag ctcacgcctg
 6181 taatcccagc actttggag gccgaagcgg gcaagatcacc cgggtcagga gttcgagacc
 6241 agcctgatca acatggagaa accccgtctc tactaaaaaa tacaaaatta gctaggcgtg
 6301 gtggcacatg ccagtaatcc cagctactt agagactgag gcaggagaat cgcttgaacc
 6361 gggaggcaga ggttgcggg agccaagatc gcgccattgc actccagcct gggcaacaag
 6421 agcaaaaactc tgtctcaaaa aaaaaaaaaacc acatacaac cagagataat attataatga
 6481 gcctccaagt gcctaccacc ttgctgcagc acttgtcaat ccaggagcca cccacctcac
 6541 cggtccccca ctcattacca ccctccctta ctcattact gaggttaatc ctggcagca
 6601 tgatcatttc tttttttct ttttatttat tttgagacag gatctgtctc tgcacccag
 6661 gctggagtgt agtggcatat ctctgctcac tgcagctct gcctccggg cagaagccat
 6721 cctcccacct cagcctacat agtagctggg accacaggca cacaccacca cacactgcta
 6781 atgtttgtat tttttgttag agactgggtt ttaccatgtt gatcaggctg gtctcaaact
 6841 cctaggctca agcaatcctc ccacccctggc ctcccaaagt gctagaatta caggcgcgag
 6901 ccactgcacc cagcgaagaa cacttttaa aaataaaata ggccggcgc ggtggctcac
 6961 acctgttaatc ccagtacttt gggagccaa ggagggcgaa tcatgaggc aagagattga
 7021 gaccatccta agtaacatgg taaaacccca ttttactac aaataaaaaa acaaaattag
 7081 cctggcgtgg tggcaggcgc ctgtagttcc agtactttgg gagctgaggc aggagaatgg
 7141 agtgaaccccg ggaggcggag cttgcagtga gctgagatca tgccactgca ctccccctg
 7201 gggcaacaga gtgagactcc caaaaaaaaaa aaaaaaaaaagcc ccccccacca acacacaata
 7261 atataaaataa ataaaatcc acaaatactat tatecacatc tacaactca acaaaaattt
 7321 cttaatatca tcaaataccca agtttgtgtt caaattttcc tgatttttc ataaatatac
 7381 tcttacagtt gttttttttt agcgagatcc aaatgagacc cacctgttga ctttgcct
 7441 tagggtttcc cagggtctga attttgttga cgacattccc atgttgcata gtaatacgg
 7501 cctccatgccc ctgtttttt ctgtaaactg atagatgtgg aggtcaatc acatttgc
 7561 ttgatattact ttggcaaaa tagttcatca gtgatactct atacttctt tgctttaca
 7621 tccggaggct gataatgtct gcttttctt cttttctaat tatttgcata aggaaaaatq
 7681 tggggggttt ggagaaaaaa acccttaagt acataactcgc taaatcacat tgctacaggt
 7741 aacttccatt aagaacttga aagtaaagg agtgcattt tcccctaggg aacacaatga
 7801 tagacaggag ctttagtcta cagcttgaag gattgtattt atacctaagc aaccctctg
 7861 gaccagttt atgttatttag ctgtgatgtt tccttacattt tgatgtcatt atccttactt
 7921 agctccctta aagcagagat caagatgaaa aggcttcag ctgcagcatg gcacatggag
 7981 attagagtgg ggcttttggc tgctgaggag cagacctaga atggaaata gatggagcc
 8041 acagaagtga aggtccccct ccctcatgca tcaacctact ccacatctcc aggtctgcac
 8101 atctgttcaag ttactgaatc ctgtgttcaagc taccttctt ttctttttt ttttatttat
 8161 ttattttattt ttttttttgag atggagttt gctttgttta cccaggctgg agtgcataatgg
 8221 tgcaatctcg gtcactgca ccctccaact cccaggttca tgcaattctc ctccccctcagc
 8281 ctcccaagta gctgggatta caggctgcac caccatgttgc ggctatattt tgaaaaatca
 8341 gttagagagag ggttccacca tggggccaa gccggctcg aactcctgc acatcaggat
 8401 ccacccaccc tggccctccaa aaatgctggg attacagggt tgagccacca tgccctgt
 8461 aaactacctt cttaaaagct cttagagagg gcttttacc ttttgcata ttttgcac
 8521 ctccgcagat ctgtatggc atctcaggat tttttttttt ttttgcac
 8581 agtgtctcac tctgtcaccctt aggattgggtt gcagtggcac gatcatgggt cattgcagcc
 8641 tccacccccc aggctcaagt gatcctctt actcagccctt ttgaatagct gagaccacag
 8701 gcttgcgtca ccatgcccag gtaatttttta atttttttt gtagaggcag ggtctcacat
 8761 tatgttgcctt agtctggctt cgagaactcc tggctcaag caatcttcc tgcctgggct
 8821 cccaaagtgg tgggattaca ggggagagcc accacaccta ggcaggagga ttttttttt
 8881 acaccaaata aaacattttt acccaaatac agttatccaa atattaaatt aacaagagtt
 8941 agggtgaccc tattaaatttag tgtaatttcc aaatagtaat gaacataatg gatagttga
 9001 gatttctgtt acttttctaa ttttgcgtt gttttttttt tttttttttt
 9061 tttttttttt gggatgttcc ctcttgcggcc gggggccatggc gttttttttt ttttttttt
 9121 ctccacccaa cctccgcctc ctgggttcaa ggcattctcc tgcctcagcc tcttgcgttag

FIG. 18 (cont.)

9181 ctgggattac aggactgtgc caccacgtcc agctaatttt gtatTTTtag tagaaacagg
9241 gtttctccat gttggtcagg ctggcttga actcccaacc tcagggcgtc cgccccccctc
9301 ggcctcccaa agtgctggg ttacaggtgt gagccacccg acctggccaa tatttgtat
9361 ttttatttgc gacaaagtca aaggttctct tcataatttt gtgtgtatc gcctacaagc
9421 ataattaaaa taaaactaa atttcagttt aaagtttact gaaaataaat atgtatTTT
9481 tattccctat ttaagcttt aatccctga cttcctatac cattaccact gtcctagttc
9541 aggttcatgt tgTTTTTAC tttaattgtt atcacagttt cttAACATTt ctccttatgt
9601 tctccagttc tgttaggtgt aaatctgacg tggtcacttc tcagcttgg atccttcaagt
9661 gcaccaccac agccttgaac tacatattt aataacatat ttatTTTca gaaactttaa
9721 actgaaattt agtgTTTATT ttaattatgc ttgtaggcga tacaccacaa taatatgaag
9781 agaacctttg actttgtcgta caataaaaag tcccttgagg ggacttcaga tgtaagtccc
9841 ttagctgttc gttaaaactc ccccaggctg acccaataca caatcttgac tttaaaccac
9901 ttgtcatctt aaatcaactag catttctgg aaaaaaaAGC cattttcct tcagggctaa
9961 gctcagggac caattctgtg tcacccctt tgaatcctga tgatattcac ttcttattt
10021 gacctgattt attggggcccc agacaccatg ctgagttt gggattcagc tctggacaat
10081 gtcaaATGTC agtcctgcct ttcaGATCCT ttctacttgg tgagccctgg agtgctgggt
10141 ctccTCGCGG tgctgcctgt gctccctcctg cagatcactc ttggcctcgt ctccctctgc
10201 ctgcagtaca gactgagagg tacagggcag agggtgggtg gatcaggatc ctTCTTTAA
10261 atgagctggc ttcttgagc tacaccactt aacatgtatt tgtagtgac ttctgggttc
10321 agaagtttt ctcaactattt agtgataaaag aaaaaaaaaa actccatgtat gaaagagttt
10381 tacatcttac ggaatgtttt catatgaata atcggacca gcatTTCCCT atgagctaaac
10441 tatGCCatAT agtaaccccc ttttacagag gatACAactG agGCCAGGAG tagttcagt
10501 acttactcaa accgatataaa ctTATAAGTG ttagAGCTGA ggCTCTGTa tcataccTAG
10561 cagETCCatG caacttggg gagtgtgac ttcaAGtCA gacaggTCTA ggCTTATTAG
10621 agTTTGAAT aaagatactg aagtggaaat ctctaccaca cagttagggcgt tcgaaaattt
10681 ttccTCTTT ctccattcaa cactgaggac tcaggTTCAg ctgctgtatga agctcctt
10741 ttTQCCTAG agctttcatt ctgagccttc tcTCTCTACC aagtgtctcc ccaatGCCAG
10801 agcaggaaga gtcTTcACTC ctcccAATGC cccacCTCCC atttGTTact aagaggagag
10861 gagaaAGTAG caaggagggt atggggatgt ttctggggg atgggtgtt gtcgcatCAA
10921 caacaAGTC ctTCTCTCA ctTGAATTc atcccAGATG ctTGTCTTGT tacttcttcc
10981 acacaaaaaa aggCCTTCAG ccCTCATGGC tgAGCAGAAA gaatCTGAAT gttAGAGTC
11041 ggcagCCTGG gttGAATTc catCTCAGGT actGAACTCT atAGCAAAAT tCTTAGATTc
11101 tccaAGCTC agttgccttG tctgtcaat agAGAAACa tcTTCTGTCC taaATTGTA
11161 ggaggattaa agtcatgcaa agtgcctact acaAAATCCAG tcacAAAGTA gctAGCTACT
11221 cactaaatgt tcagctcctc cctcctcatt cagatggaa gtagcggaa gtggctttag ataaacAAAG
11281 tggcaACGCA gtgggctggc gcagctctgt gaaCTGAGAA tCCAAGAAAAA ggggCgAAAGA
11341 gcagCTGGGta tgTATTGGat gcttGtGtGtG gcttggagca ttGCTCACAT tCTTATTCTG
11401 ctattgtatc tagactataAG ctAGAGAAAG agccgcaacc atTGGCTTA aatCCAGTGC
11461 tcttcctact ctccTGTAGGT tgTTTCCAGG ctgcagAGAA atAGCCTGCA caaggGGGCCCC
11521 aggCCTGGG tgTGGGAGGG tccccaccGA gagCCAGAAAC atGCAGGAAC taaaATGTTG
11581 ccttttctA ttttaggaaa acttcgagca gagatAGGTG agttccAGTC atCGTTCTC
11641 ccaattttG ccttttGtt ttttGcata acggAAATGG tccCTATTCT ggacGGTGT
11701 tccctctcaa taccctgtt tcccCTCAGT tccCTTTCTC ctacAGTGGG tgTGTCTG
11761 ctagaacaAG tttaAGTAA ttaAAATAACa aagactcagg ataaaAGGAT ctttttGGA
11821 gtgcCCTACT aaatCCATT ccattGTTt ctctttcaga gaaATCTCCAC cggactTTG
11881 gtaagtTCCG gcatgtctag gcccTCCCAG gtcAACTTGG tatttcaCTC tagTTCCAGT
11941 cacCTGGGG aacaaggacc cctggctcct ggttGAGTCC ttccCTCTCT tctcttttCT
12001 ttctttAAAT aagaAGTCA tTGCAATTAG gatTTGTTAA atcataataa zaataACTCAT
12061 gtactgtttt tatgtGCCAG gcactattt aactacttA caaaaACGTT atcttatttCT
12121 gtttaactcc ttatgcacat gatctcttt ttcAGGAATG ccaAAACACa ggtAAATAGA
12181 tcgtttacac gtaaaacctga tGTCGTGGTg gggaggtgaa acaaAcAGAA acaAGACACA

FIG. 18 (cont.)

12241 actgtatcac ctgtacttat atttctgctt tacaactca ggatgttcc atgagtacag
 12301 aacatgacta atcagagaag acctcataga ggaatagaaa agccaccaag cccactagg
 12361 aattgacccc tcaaggacat ggtttctagc cttttgttc actgcagatt gcccaatgcc
 12421 taaagataat ggcaacagaa gagcacccaa atatttgtt gataaatgtt gcagacacta
 12481 gaaggtgtca ttagggcaca gatggtacct tctctgagca aacttccttc acagctcctc
 12541 ctcccggaggc tgttagtgac tctactcttg tcacctggca cacagagttc tatcgtaacga
 12601 ttaggaaat tagaccatg tgtaggaccac acacacacac atcttacac acccaaagag
 12661 gaggaatagt atcttgttt tgaggactt gactatgaaa ggtcttaact ccttttgta
 12721 ccatgaatct ctctggcact ccagtgaagt ctaaaggacc ctttgcaaa atgttttaa
 12781 atatacacat aaaatagaac acataggatt gaaaaacaa tcattgtact aaaatacaat
 12841 tatcaaccga taatcacat tgtgatatacg taacataaaat gtttctttt ttttttttg
 12901 gaggcagat ttggcttgc tcacccaggc tgtagtgcaaa tgccgcgtc taggtcact
 12961 gaaacctctg cttccgggt tcaagcgat ctcagcctcc tgagtagctg ggattacagg
 13021 tgccccccac cacaccaggc taattttgt atttttagta gagacttagt ttcaccagg
 13081 tggccaggct ggcctcgaac tcctgaccc aggtgatcca cctgccttgg cctccaaag
 13141 tgctggatt acgggcatga gccaccgtgc ccggccataa atatttctt agccaaagta
 13201 atacattaag taatgttagca gcaagtctaa taacctgtaa ttcttctt tctttctt
 13261 ttctttttt tttgagatga agttttttt agatggagtg caatggcaca atctcggtc
 13321 actgcaaccc ccacccctg ggttcaagcg atttcctgc ctcagcctcc caagttgctg
 13381 gaactacagg cgcatgccac catgcccagc taattttgtt atttttagta gagacggggt
 13441 ttcaccatgt tggccaggct ggtcttgaac ccctgaccc aggtgatctg cctgccttgg
 13501 cttccaaag tgctggattt acaggcatga gcoaccaggc ccagccaaat aaccttaat
 13561 ttcaacatac taataaacat aaacagtatt tcaagatttc tgcaataact ctaatggaa
 13621 tggaaaacatc tggcttcc attggtaatt aagtccacagg tactgctcat attgtggta
 13681 gttgtaaaat gttttgggtt gttttgttt ttccaagact tggggaaatg ggtgttggtg
 13741 ggtcaacaa gagtcttgct ctgtggccca ggctggatgt cagggcagg atcttggctc
 13801 actgcaaccc cccctccca ggttcaagcg atttcctgc ctcagcctcc tgagtagctg
 13861 gcattacagg catgtccac cacgcccagc taatttttac atttttagta gagatggggt
 13921 ttcaccatgt tggcttggct ggtcttgaac tcttggcctc atgatccacc cgtctcgac
 13981 tcccgaggat tgggatttcc aggcatgagc caccacaccc ggcaggatgtt acattttaa
 14041 tggaaaagaaaa tggtaatcc agtttattttt aataaggagg cagtactttt ctcattccaaag
 14101 ttcattggact ttctgttattt tggcccaaga gtccttttgtt gttcttagac cccaggtaa
 14161 ggaacccaaa aagacaggat ggtggggcat gaggggggac acatgttaat ccctgtt
 14221 tctggtaac aattccatgtt ccacttttctt gagggtgccc tgctggaaataaccctgtt
 14281 tggtaatttttcc cgggttctt gaccctttgtt tgcccttgc atctgtaca actggctaca
 14341 tcgaagacta gcagggtcag tggctggca gcaggcaaga ccacccaaata gtgggggac
 14401 aagtccatgt tggatggaa gccaaaagag aatagaacca ggactcaaga tttaggggagc
 14461 tgggatttcc ttattccatgtt gtccttgc ccaaccccaag gctcttgc gaaactgtga
 14521 agagaaccac ttactggatc tggggatcc cccagtgaa agggcaggatgtt gggtcactcc
 14581 aaatgtccat agggaggatg tggggaaatgtt gctattccatc ttccactaat cacatattt
 14641 ttctttttt ttttcaggcc aattccatgtt agagctacgt aagttctt ctctctgtt
 14701 taagcagaga ataaaaagcc agggaaaggaa gacagaagca acaagaggaa gaggcggggt
 14761 attgagggat cacattccca gggaaaaggaa ggagctggaa agcctgggtt gaggaaagac
 14821 tcctctgggg aggttagaggg caaagaagcc agctgttgc gacacattt caggtggcag
 14881 agaagctgga ggcacttca tctgcccacat gatccattcc tccttcactg cccctaagca
 14941 ggaatccaaac ccttagtggc tctcattgccc attccacacgc aactgcccag tgcctcacct
 15001 ctcaagatcaa ccattggagc agggatggag acaaagatgac cccaaaggct ttcttcct
 15061 cttagtcaat ggtttatgtt tacaatgtt tgacatacg ttttcaggat attttctt
 15121 tcttccttgc aatcccttgc ggtgtatgc acatcttggc aggggtggag gagagccctgg
 15181 ttggcccgaggg atttgtccctt ggggacatctt catccatcaa ttgcacact cactggcattc
 15241 ttgtctatgg ggacattcca atttgcattt ctaggaacac tctgaattcc aagttagaaatt

FIG. 18 (cont.)

15301 gatttccctt cttctgtcat ctacctttc tcttcatttt cccatTTTta ttacccttct
 15361 ttccatTTtct ctctccagtc ttccacCTgg aagCCCTCtc tggctaagga caggcaggTg
 15421 cccCTCTCtc catcagagga cacCTgtact ggagagcaac acaggatggT ctctgcCATg
 15481 aactggagGC caggaatCTC cTCactgaaa attacagtat ggtaactttg caaatggTgg
 15541 ttgtttCTCtc caagactcca gcccTgattg cgcaaaACTg aaaggcatgt gaagggaagg
 15601 aagagGAAGA gtgcaAAACA ttGAAGAGAG agCTgAGTgA gCTgAAGAGT gaggatATgA
 15661 gtagccccAA cccAAACCTg gagatGGGGA gAAACCTACA gaataCTAGC cAGAGCTCT
 15721 cCTTGTCTG gcAGCCTACT agggacTgg ggaAGCAAAA acgAAAGCTg ggcaACATgC
 15781 ctgCTTtaga atgtttCCTt tCTacttaca catCTTCCAC aggtCTCAGA atCTTtCCTt
 15841 CCTCTCATCC tTTTCTCCTA tCTacatATC tatCAGAGTA tCCACTGTTt ATTCAACAAAC
 15901 tactacttga tggtcagaca caAAACAAACA agCTAGGTgC taattaATAA agatacAGT
 15961 tttggccGGG tgCGGTGGCT CACGCCtGta ATCCCAGCAC tttgggAGGC CGAGGCGGGC
 16021 gaatcacGAG gTCAGGAGTT CAAGACCAGC CTGGCCAACA tggTgAAACC CCATCTCTAC
 16081 taaaaataca aacaATTAAAC tgAGCAGATg ggtGGGcACC tataATACCA gCTACTCCG
 16141 aggCTGAGGC aggAGGAATCG CTTGAACCCCA ggAGGCGAGAG gTTGCAgTgA gCTGAGATgC
 16201 CGCCACTGCA CTCTAGCCGG AGTgACAGAG TAAGACTCTG TCTCAAAAT AAATAAATAA
 16261 aaaaATAAAT AAATAAATAA ATAATAAAATAA ATAATAATAA CAAGTTTCA TAAGCACACT
 16321 tctaACCCCT tGTCTTtAT GTATTTCTt CCTTATCCAC GCACCTGTCT CCCTCTACTC
 16381 cAGCCTCATT ACCCCAGAGG TCAGTCTCA ggAAAActAA ACACAAAGAA AGAGCTCAGT
 16441 cAGAAAGGGCC ATTtATTtAT GTTCAAGAT GCTCACTGCC TCTTTGTT TGTCTCTTt
 16501 gCAGGCCtTC tCTCTTAGGC CTCTTCTCT gggGGTATGG ATCTGGGGG GAGATRGATC
 16561 ACCTCCATgC TTCAATTCTC CCCCAGGCAt AGTGGGGACa TCAgAGAGA AGCCAAGCCA
 16621 CTGGCCCCAGG ATCACCCGGC ATTtATGGTg GCTGCTCTG cacAGGTCTC TGCCTTATA
 16681 GCCCCTCCAG TGATCCATAA GGCCCTCTTt CTCCCCAAAG gagAGGTcAC agatAGGGCA
 16741 aaggtagCTC ttCTGCTTC AGTGGGTCTG CTGGTGTCTG accAGCCTGG AAAATGAGCT
 16801 gaaAGACTtG CTGCAATGGA AGCAGTAGtT gggCGGCTt GTGAGGTGGC CCTTCTGGT
 16861 tCTGGAGAGA TAGGATTtCT TGCTAAAGT CAAAGAACAA TGGGGGCAAC agaAGAGACATT
 16921 gAGTCTTgAG ggCTTCACTG GATGAGAGTt GGATCTGGCA TCCtGACAGA GGGTTCCAGT
 16981 gATGGGTGCC TGGGTCTGG TCAcAGGTGc TTGGTTCTTA AGTACAGATG CCTGGTTCTG
 17041 ggCCATAGGA CCCTCAGTtC TAAATATGGG TTCTGGGAC CTGGCCACTG GTGCAtGGTT
 17101 cacATCCAAA AGCCCTGGA TGGACCTCTG GCTTCTGGG ATGGGTGTCT GGAATTCAgC
 17161 CTGGGTGCTt ggaATCCTCA AAGTACACTC CTGGTTtCCA TCCACTGGCT CCTGGTTtG
 17221 gTGTATCTC tggTGGCGTT TGAgtCTCAGA CTGGTCCCGG AAGCTCTCC CACACACAGA
 17281 gCATGAATGG gGCCGgTAAC CCAGATGGAC GCGGCGGTGA CGACTTAGTC CAGAAGCATC
 17341 acAGTAGGTG TTGTCAcAGA GCGTgCAACA GAAGGGCCTC TCCCCAAGAT GCAgTCGtCT
 17401 gTGATAGCTG AGGGACTTGG GGCTCCGAAA CAACTTCCCA CACTgACTgC AGCTGTtAGT
 17461 cAGCTTGGGA TTGTGAACAA ACTGGTGGCT ATAGAGGTAG GAGCGCCTgC TgAAACATTt
 17521 ggcACAGGTG TAGCAAAA

FIG. 18 (cont.)

1 tttgtatgtc attgcaggat tcatgcttc cagtgtgtca tctatggaac tgcctcttc
61 ttcttccttt atggggccct cctgctggct gagggcttct acaccaccgg cgctgtcagg
121 cagatcttg gcgactaca gaccaccatc tgccggcaagg gcctgagcgc aacggtaaca
181 gggggccaga aggggagggg ttacagaggc caacatcaag ctcattctt ggagcggtg
241 tgtcattgtt tggaaaaatg gctaggacat cccgacaagg tgatcatcct caggatttg
301 tggcaataac aagggtggg gggacaa

FIG. 14

1 ctgtatcagt gctcctcgtc gcctcaactgt acttcacgga agagacttgg ttgactggcc
 61 acttggagcg gaatcaggag acatccccaa ctcagagaga ctgagcccta gctcgcccac
 121 ttgctggaca agatgatatt cttaccacc ctgcctctgt tttggataat gatttcagct
 181 tctcgaggggg ggcactgggg tgcctggatg ccctcgatcca ttcagccctt cgagggcacg
 241 tgtgtctcca tcccctgccc ttcgacttc cccgatgagc tcagaccggc tgggtacat
 301 ggcgtctggt atttcaacag tccctaaaaa aagaactacc cggcagtggt cttcaagtcc
 361 cgcacacaag tggtccacga gagcttccag ggccgtagec gcctgttggg agacctggc
 421 ctacgaaaact gcaccctgtc ttcagcacc ctgagccctt agctgggagg gaaataactat
 481 ttccgaggtg acctgggccc ctacaaccag tacaccttct cggagcacag cgtcctggac
 541 atcatcaaca cccccaacat cgtggtcccc ccagaagttt tggcaggaac ggaagtagag
 601 gtcagctgca tgggccggg caactgccc gagctgcgcc ctgagctgag ctggctggc
 661 cacgaggggc taggggagcc cactgtctg ggtcggtgc gggaggatga aggacactgg
 721 gtgcagggtt cactgctaca ctgcgtgcct actagagagg ccaacggcca ccgtctggc
 781 tgtcaggctg ccttcccaa caccacccat cagttcgagg gttacgcag tctggacgtc
 841 aagtacccccc cggtgattgt ggagatgaat tcctctgtgg agggcattga gggctcccac
 901 gtcagcctgc tctgtggggc tgacagcaac cccgcacccgc tgctgacttg gatgcggat
 961 gggatgggtg tgagggaggg agttgtcgag agcctgtacc tggatctgga ggaggtgacc
 1021 ccagcagagg acggcatcta tgctgcctg gcaagagaatg cctatggcca ggacaaccgc
 1081 acgggtggagc tgagcgtcat gtatgcacct tggaagccca cagtgaatgg gacgggtgt
 1141 gcggttagagg gggagacagt ctccatcctg tttccacac agagcaaccc ggaccctatt
 1201 ctcaccatct tcaaggagaa gcagatcctg gccacggtca tctatgagag tcagctgcag
 1261 ctggaaactcc ctgcagtgcac gcccgggac gatggggagt actgggtgt agctgagaac
 1321 cagtatggcc agagagccac cgccttcaac ctgtctgtgg agttgtcc cataatcc
 1381 ctggaatcgc actgtgcagc gcccagagac accgtgcagt gcctgtgt ggtaaaatcc
 1441 aacccggaaac cttccgtggc ctttgcgtc cttccgcac acgtgactgt gaacgagaca
 1501 gagagggagt ttgtgtactc agagcgcagc gcccctctgc tcaccagcat cctcacgctc
 1561 cggggtcagg cccaaagcccc accccgcgc atttgtactt ccaggaacct ctacggcacc
 1621 cagagcctcg agctgccttt ccaggagca caccgactga tggggccaa aatcgccct
 1681 gtgggtgtc tggtgcctt tgccatcctg attggcattt tctgtacat caccagaca
 1741 agaagaaaaa agaacgtcac agagagcccc agttctca agggagacaa ccctcatgtc
 1801 ctgtacagcc ccgaattccg aatctcttgc gcacctgata agtatgagag tgagaagcgc
 1861 ctggggtccg agaggaggct gctgggcctt agggggggac ccccaagaact ggacctcagt
 1921 tattccact cagacctggg gaaacgaccc accaaggaca gctacaccct gacagaggag
 1981 ctggctgagt acgcagaaat ccgagtcag tga

FIG. 20

1 masqkrpsqr hgskylatas tmdharhgfl prhrdtgild sigrfggdr gapkrgsgkd
61 shhpartahy gslpqkshgr tqdenpvvhf fknivtprrtpppsqgkgrgl slsrfswgae
121 gqrpgfgyygg rasdyksahk gfkgvdaqgt lskifklgr dsrsgspmar r

FIG. 24

1 mgalleccarc lvgapfaslv atglcffgva lfcgcgheal tgtekliety fsknyqdyey
61 linvihafqy viygatasfff lygalllaeg fyttgavrqi fgdyktticg kglSATVTGG
121 qkgrgsrgqh qahslervch clgkwlgpdc kityaltvvw llvfacsavp vyiyfntwtt
181 cqsiafpskt sasigslcad armygvlpwn afpgkvvcgsn llsicktaef qmtfhlfiaa
241 fvgaatlv nfavlkimgt gatkf

FIG. 26

1 maslrsrpslp sc1csf1111 llqvsssyag qfrvigprhp iralvgdeve lpcrispgkn
61 atgmevgwyr ppfsrvvhly rngkdqdgdq apeyrgrtel lkdaigegkv tlrirnvrfs
121 deggftcffr dhsyqeeaaam elkvedpfyw vspgvivvlla vlpvllqit lglvf1clqy
181 rlrkgklraei enlhrtfdph flrvpcwkit lfvivpvlgp lvaliicynw lhrrlagqfl
241 eelrnnpf

FIG. 29



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(54) Title: METHODS FOR AMYLOID REMOVAL USING ANTI-AMYLOID ANTIBODIES

(57) Abstract

Methods and related immunoglobulin peptides and fragments thereof are disclosed that enhance the cell-mediated immune response of a patient to deposits of amyloid fibrils. These methods exploit the opsonizing effect of antibodies directed toward amyloid material or its component parts.

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**Title: METHODS FOR AMYLOID REMOVAL USING ANTI-AMYLOID
ANTIBODIES**

5

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10 This invention was made with government support under Grant No. 2 R01 CA 20056, awarded by The National Institutes of Health. Thus, the government may have certain rights in this invention.

Technical Field

15 The present invention generally relates to methods for treating amyloid-related diseases. Specifically, the present invention provides therapeutic antibody-related methods to effect the removal of amyloid fibrils by a patient's own immunophagocytic system.

20 **Background of the Invention**

Amyloidosis refers to the pathological deposition of proteins in the form of congophilic, green birefringent fibrils, when congo red-stained, either dispersed or in the form of localized amyloidomas. Such deposits are symptomatic of several diseases, for example Alzheimer's Disease, inflammation-associated amyloid, type II diabetes, bovine 25 spongiform encephalopathy (BSE), Creutzfeld-Jakob disease (CJD), scrapie and primary amyloidosis.

Amyloidoses are generally categorized into three groups: major systemic amyloidoses, major localized amyloidoses, and miscellaneous amyloidoses. Major systemic amyloidoses include: chronic inflammatory conditions (*e.g.*, tuberculosis, 30 osteomyelitis, *etc.*); non-infectious conditions such as juvenile rheumatoid arthritis, ankylosing spondylitis and Crohn's disease, *etc.*; familial Mediterranean Fever, plasma

cell dyscrasia (primary amyloidosis) and various familial polyneuropathies and cardiomyopathies. Major localized amyloidoses include: chronic dialysis usually for greater than 8 years, Alzheimer's disease, Down syndrome, Hereditary cerebral hemorrhage (Dutch), and non-traumatic cerebral hemorrhage of the elderly.

5 Miscellaneous amyloidoses include: familial polyneuropathy (Iowa), familial amyloidosis (Finnish), hereditary cerebral hemorrhage (Icelandic), CJD, Medullary carcinoma of the thyroid, atrial amyloid, and diabetes mellitus (insulinomas). Other amyloidoses include those referenced in Louis W. Heck, "The Amyloid Diseases" in Cecil's Textbook of Medicine 1504-6 (W.B. Saunders & Co., Philadelphia, PA; 1996).

10 Transmissible spongiform encephalopathies which cause CJD and Gerstmann-Strässler-Scheinker (GSS) disease are described by B. Chesebro *et al.*, "Transmissible Spongiform Encephalopathies: A Brief Introduction" in FIELD'S VIROLOGY 2845-49 (3rd Edition; Raven Publishers, Philadelphia, PA; 1996) and in D.C. Gajdusek, "Infectious amyloids: Subacute Spongiform Encephalopathies as Transmissible Cerebral

15 Amyloidoses," 2851-2900 in FIELDS VIROLOGY (1996). Many of these diseases are likely mediated by prions, an infectious protein. See S.B. Prusiner, "Prions" in FIELDS VIROLOGY 2901-50 (1996) and the references contained therein. The inherited forms of amyloidoses as described on Online Mendelian Inheritance in Man (OMIM) "www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?" Each of the above is incorporated

20 herein by reference.

Very rarely do patients with clinically proven amyloidosis spontaneously achieve complete remission, perhaps because the amyloid fibrils themselves are non-immunogenic. Various therapies for amyloidosis have been investigated, such as high-dose chemotherapy, steroids, iodinated doxorubicin, and stem cell replacement therapy.

25 However, in only one type of amyloid disease, Familial-Mediterranean amyloidosis, has drug treatment (with colchicine) been shown to be effective.

The use of monoclonal antibodies (mAbs) to induce or modulate the immunological removal of an otherwise unrecognized entity is known. mAbs have been successfully used in treating non-Hodgkins lymphoma and breast cancer, for example.

30 Previously, a variety of studies have characterized antibodies that bind to amyloid proteins or amyloid fibrils. See, for example, U.S. Patents Nos. 5,714,471; 5,693,478;

5,688,651; 5,652,092; 5,593,846; 5,536,640; 5,385,915; 5,348,963; 5,270,165; 5,262,332; 5,262,303; 5,164,295; and 4,782,014. In addition, several publications have suggested that anti-amyloid antibodies might be useful for studying the progression of beta-amyloidosis and for various therapeutic options. See, for example, Bellottii *et al.*, 5 *Scand. J. Immunol.* (1992) 36(4):607-615; Bellotti *et al.*, *Ren. Fail.* (1993) 15(3):365-371; Walker *et al.* *J. Neuropathol. Exp. Neurol.* (1994) 53(4):377-383; and Bickel *et al.*, *Bioconjug. Chem.* (1994) 5(2):119-125. However, no therapeutic antibody has been demonstrated to halt or reverse the deposition of amyloid fibrils in a patient. Thus, a need exists for a method for treating amyloidoses using antibody formulations containing 10 antibodies that bind to amyloid fibrils.

Summary of the Invention

The present inventors have discovered new methods of treating amyloid-related diseases and conditions. These methods exploit the opsonizing effect of mAbs directed 15 toward the protein constituents of amyloid.

The present invention includes a method of treating a patient having an amyloid-associated disease comprising the step of administering to the patient a therapeutically effective dose of at least one immunoglobulin polypeptide, or fragments thereof, together with a pharmaceutically acceptable carrier; wherein the immunoglobulin polypeptide or 20 fragment thereof, may be a substantially purified immunoglobulin polypeptide that binds to a human amyloid fibril, wherein binding of the polypeptide opsonizes the amyloid fibril.

In particular, the present invention relates to the use of any one of, or a combination of, the three monoclonal antibodies discussed below. These antibodies have 25 general anti-amyloid binding properties and provide an extrinsic opsonizing reagent that activates a patient's own cellular immune clearance mechanism.

Brief Description of the Drawings

Figures 1A and 1B. Figures 1A and 1B are reproduced photographs of a Balb/c 30 mouse just after an injection of amyloid is made (1A) and 14 days after the injection

(1B). The injection site was shaved to better illustrate the "hump" caused by the injection of the amyloid material.

Figures 2A-2B. Figures 2A and 2B are reproduced photographs of human neutrophils (multi-lobed nuclei) adhering to human amyloid opsonized *in vitro*.

Figures 3A-3D. Figures 3A-3D are reproduced photographs of immunohistochemically stained amyloid-laden tissue samples (20X magnification). Figure 3A is a tissue sample from a patient with κ 1 amyloidosis stained with Congo red; the amyloid deposits, viewed under polarized light, appear as blue-green particles.

Figure 3B is a tissue sample stained with alkaline phosphatase after labeling with anti- κ I (57-18-H12) mAb. Figure 3C is a tissue sample stained as in Figure 3B, but with anti- κ IV (11-1F4) mAb. Figure 3D is a tissue sample stained as in Figure 3B, but with anti- λ VIII (31-8c7) mAb.

15

Figure 4. Figure 4 is a reproduced photograph showing a fluoresceinated (FITC) κ 4 mAb bound to human amyloid implanted into a Balb/c mouse. The mAb was injected into the thigh of the mouse. The amyloidoma was excised 72 hours post injection and viewed using an epifluorescence microscope (20X magnification).

20

Modes of Carrying Out the Invention

General Description

The present invention utilizes immunoglobulin polypeptides to modulate and to enhance the degradation and removal of undesired deposits of amyloid fibrils in a host or patient. It is envisioned that the invention will be used, for example, to treat humans suffering from a disease or condition characterized by an undesired deposition of amyloid fibrils. Without intending to be bound by any particular mechanism of action, it is believed that the administration of immunoglobulin peptides according to the present invention opsonize the deposited amyloid fibrils in a patient suffering from amyloidosis, thereby assisting in their removal from the patient by the patients' own immune system. It is believed that the patient's immune system alone is unable to remove the amyloid

fibrils in conditions modulated by amyloid fibrils without such a therapeutic intervention, presumably because the amyloid fibrils are themselves relatively non-immunogenic.

To treat a patient with amyloidosis, a therapeutically effective dose of 5 immunoglobulin polypeptide or fragment thereof according to the present invention is administered together with a pharmaceutically suitable carrier or excipient. Upon the binding or adhering of such immunoglobulin polypeptides to undesired deposits of amyloid fibrils, the latter are believed to be opsonized.

Single or multiple administrations of the compositions of the present invention 10 can be carried out in dosages and by administration protocols known to those skilled in the art for the administration of other therapeutic antibody products. These parameters may be selected and/or optimized by the physician treating a particular patient.

Preferably, a therapeutically effective dose of a pharmaceutical formulation of the present invention should deliver a quantity of anti-amyloid immunoglobulin polypeptide 15 sufficient to substantially inhibit the undesired deposition of amyloid fibrils or to substantially inhibit the rate of any undesired deposition of amyloid fibrils. More preferably, the formulations should reduce the overall burden of deposited amyloid fibrils in a patient. Further, administration of such formulations should begin shortly after diagnosis of amyloidosis and continue until symptoms are substantially abated and 20 for a period thereafter. In well established cases of disease, loading doses followed by maintenance doses may be required.

Definitions

The terms "peptide," "polypeptide" or "protein" are used interchangeably herein. 25 The term "substantial identity," when referring to polypeptides, indicates that the polypeptide or protein in question is at least about 30% identical to an entire naturally occurring protein or a portion thereof, usually at least about 70% identical, and preferably at least about 95% identical.

As used herein, the terms "isolated," "substantially pure" and "substantially 30 homogenous" are used interchangeably and describe a protein that has been separated from components which naturally accompany it. A substantially purified protein will

typically comprise over about 85% to 90% of a protein sample, more usually about 95%, and preferably will be over about 99% pure. Protein purity or homogeneity may be indicated by a number of means well known in the art, such a polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band on 5 a polyacrylamide gel upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized.

Proteins may be purified to substantial homogeneity by standard techniques well known in the art, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for 10 instance, Scopes, Protein Purification: Principles and Practice, Springer-Verlag: New York (1982), which is incorporated herein by reference.

Antibody purification techniques are well known in the art. Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1988), 288-318, which is incorporated herein by reference, describes, for 15 example, purification using ammonium sulfate precipitation, caprylic acid, DEAE, hydroxyapatite chromatography, gel filtration chromatography, protein A beads, and immunoaffinity.

Nucleic acids, as used herein, may be DNA or RNA. When referring to nucleic acids, the term "substantial identity" indicates that the sequences of two nucleic acids, or 20 designated portions thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98% to 99.5% of the nucleotides.

Alternatively, substantial nucleic acid sequence identity exists when a nucleic 25 acid segment will hybridize under selective hybridization conditions, to a complement of another nucleic acid strand.

"Substantially complementary" similarly means that one nucleic acid hybridizes selectively to, or is identical to, another nucleic acid. Typically, selective hybridization will occur when there is at least about 55% identity over a stretch of at least 14-25 30 nucleotides, preferably at least about 65% identity, more preferably at least about 75%,

and most preferably at least about 90% identity. See M. Kanehisa Nucleic Acids Res. 12:203 (1984), which is incorporated herein by reference.

Stringent hybridization conditions will typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and preferably less than about 5 200 mM. Temperature conditions will typically be greater than 22° C, typically greater than about 30° C and preferably in excess of about 37° C. As other factors may dramatically affect the stringency of hybridization, including base composition and size of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure 10 of any one alone.

“Isolated” or “substantially pure,” when referring to nucleic acids, refer to those that have been purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, and others well known in the art. 15 See, F. Ausubel, *et al.*, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1987), incorporated herein by reference.

A nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, 20 operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

Techniques for nucleic acid manipulation, such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labelling probes, DNA hybridization, and so on are described generally, for example in Sambrook *et al.*, (1989) 25 Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, or Ausubel *et al.*, ed. (1987) op. cit., both of which are incorporated herein by reference.

“Expression vectors,” “cloning vectors,” or “vectors” are often plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors 30 may replicate autonomously, or they may replicate by being inserted into a genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will

have an origin of replication or autonomous replicating sequence ("ARS") that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, *e.g.*, in *E. coli* for cloning and construction, and in a mammalian cell for expression.

5 Mammalian cell lines are often used as host cells for the expression of polypeptides derived from eukaryotes. Propagation of mammalian cells in culture is per se well known. See, Tissue Culture, Academic Press, Kruse and Patterson, ed. (1973), incorporated herein by reference. Host cell lines may also include such organisms as bacteria (*e.g.*, E. coli or B. subtilis), yeast, filamentous fungi, plant cells, or insect cells,
10 among others.

"Transformation" refers to the introduction of vectors containing the nucleic acids of interest directly into host cells by well-known methods. Transformation methods, which vary depending on the type of host cell, include electroporation; transfection employing calcium chloride, rubidium chloride calcium phosphate, DEAE-
15 dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent); and other methods. See generally, Sambrook *et al.*, (1989) op. cit. Reference to cells into which the nucleic acids described above have been introduced is meant to also include the progeny of such cells.

As used herein, "immunoglobulin polypeptide" refers to molecules that are
20 derived from native immunoglobulins (*e.g.*, antibodies) that have specific immunoreactive activity against a particular target, *e.g.*, against amyloid fibrils. Antibodies are typically tetramers of immunoglobulin polypeptides. As used herein, the term "antibody" also refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. Immunoglobulin genes include those
25 coding for the light chains, which may be of the kappa or lambda types, and those coding for the heavy chains. Heavy chain types are alpha, gamma, delta, epsilon and mu. The carboxy terminal portions of immunoglobulin heavy and light chains are constant regions, while the amino terminal portions are encoded by the myriad immunoglobulin variable region genes. The variable regions of an immunoglobulin are the portions that
30 provide antigen recognition specificity. In particular, the specificity resides in the

complementarity determining regions ("CDRs"), also known as hypervariable regions, of the immunoglobulins.

The immunoglobulins may exist in a variety of fragment forms including, for example, Fv, Fab, F(ab''), F(ab')₂, SvFv and other fragments, as well as single chains 5 (e.g., Huston, *et al.*, Proc. Nat. Acad. Sci. U.S.A., 85:5879-5883 (1988) and Bird, *et al.*, Science 242:423-426 (1988), which are incorporated herein by reference). (See, generally, Hood, *et al.*, "Immunology," Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, *Nature*, 323:15-16 (1986), which are incorporated herein by reference).

Single-chain antibodies, in which genes for a heavy chain and a light chain are combined 10 into a single coding sequence, may also be used. Immunoglobulin polypeptide also encompasses a truncated immunoglobulin chain, for example, a chain containing less constant region domains than in the native polypeptide. Such truncated polypeptides can be produced by standard methods such as introducing a stop codon into the gene sequence 5' of the domain sequences to be deleted. The truncated polypeptides can then 15 be assembled into truncated antibodies. Antibodies as used herein also include bispecific antibodies which can be produced such as by the methods described in the following references: Glennie *et al.*, J. Immunol., 139:2367-2375 (1987); Segal *et al.*, Biologic Therapy of Cancer Cancer Updates 2(4):1-12 (1992); and Shalaby *et al.*, J. Exp. Med. 175:217-225 (1992).

20 "Monoclonal antibodies" may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, Kohler and Milstein, Eur. J. Immunol. 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods 25 well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

Monospecific and bispecific immunoglobulins may also be produced by 30 recombinant techniques in prokaryotic or eukaryotic host cells.

"Chimeric" antibodies are encoded by immunoglobulin genes that have been genetically engineered so that the light and heavy chain genes are composed of immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to 5 human constant (C) segments. Such a chimeric antibody is likely to be less antigenic to a human than antibodies with mouse constant regions as well as mouse variable regions.

As used herein, the term chimeric antibody also refers to an antibody that includes an immunoglobulin that has a human-like framework and in which any constant region present has at least about 85%-90%, and preferably about 95% polypeptide 10 sequence identity to a human immunoglobulin constant region, a so-called "humanized" immunoglobulin (see, for example, PCT Publication WO 90/07861, which is incorporated herein by reference). Hence, all parts of such a "humanized" immunoglobulin, except possibly the complementarity determining regions (CDRs), are substantially identical to corresponding parts of one or more native human 15 immunoglobulin sequences. Where necessary, framework residues may also be replaced with those within or across species especially if certain framework residues are found to affect the structure of the CDRs. A chimeric antibody may also contain truncated variable or constant regions.

The term "framework region," as used herein, refers to those portions of 20 immunoglobulin light and heavy chain variable regions that are relatively conserved (*i.e.*, other than the CDRs) among different immunoglobulins in a single species, as defined by Kabat, *et al.*, (1987); Sequences of Proteins of Immunologic Interest, 4th Ed., U.S. Dept. Health and Human Services, which is incorporated herein by reference). As used herein, a "human-like framework region" is a framework region that in each existing chain 25 comprises at least about 70 or more amino acid residues, typically 75 to 85 or more residues, identical to those in a human immunoglobulin.

Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably from immortalized B-cells. The variable regions or CDRs for producing the chimeric immunoglobulins of the 30 present invention may be similarly derived from monoclonal antibodies capable of binding to the human type amyloid, and will be produced in any convenient mammalian

system, including mice, rats, rabbits, human cell lines, or other vertebrates capable of producing antibodies by well-known methods. Variable regions or CDRs may be produced synthetically, by standard recombinant methods, including polymerase chain reaction ("PCR") or through phage-display libraries. For phage display methods, see for example, McCafferty *et al.*, Nature 348:552-554 (1990); Clackson *et al.*, Nature 352:624-628 and Marks *et al.*, Biotechnology 11:1145- 1149 (1993). Suitable prokaryotic systems such as bacteria, yeast and phage may be employed.

Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the 10 American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference).

In addition to the chimeric and "humanized" immunoglobulins specifically described herein, other substantially identical modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known 15 to those skilled in the art. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as PCR and site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and S. Roberts *et al.*, Nature 328:731-734 (1987), both of which are incorporated herein by reference).

Alternatively, polypeptide fragments comprising only a portion of the primary 20 immunoglobulin structure may be produced. For example, it may be desirable to produce immunoglobulin polypeptide fragments that possess one or more immunoglobulin activities in addition to, or other than, antigen recognition (e.g., complement fixation).

Immunoglobulin genes, in whole or in part, may also be combined with 25 functional regions from other genes (e.g., enzymes), or with other molecules such as toxins, labels and targeting moieties to produce fusion proteins (e.g., "immunotoxins") having novel properties. In these cases of gene fusion, the two components are present within the same polypeptide chain. Alternatively, the immunoglobulin or fragment thereof may be chemically bonded to the toxin or label by any of a variety of well-known 30 chemical procedures. For example, when the label or cytotoxic agent is a protein and the

second component is an intact immunoglobulin, the linkage may be by way of heterobifunctional cross-linkers, e.g., SPDP, carbodiimide, glutaraldehyde, or the like.

Suitable labels include, for example, radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescers, chemiluminescers, magnetic particles. See, for 5 examples of patents teaching the use of such labels, U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241, all of which are incorporated by reference.

10 Immunotoxins, including single chain molecules, may also be produced by recombinant means. Production of various immunotoxins is well-known with the art, and methods can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe *et al.*, Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982); E. Vitetta, Science (1987) 238:1098-1104; and G. Winter and C. Milstein, Nature (1991) 349:293-299; all incorporated herein by reference.

15 Additional techniques for preparing immunoglobulins and immunoglobulin fragments are described in V.S. Malik *et al.*, Antibody Techniques (Academic Press, 1994); C.A.K. Borrebaeck, Antibody Engineering: Breakthroughs in Molecular Biology (Oxford Univ. Press, 1995); and P.J. Delves *et al.*, Antibody Production: Essential Techniques (John Wiley & Sons, 1997), which are incorporated herein by reference.

20 "Opsonize", as used herein, refers to the binding of an immunoglobulin polypeptide to a particular target, particularly epitopes found on deposits of amyloid fibrils, such that the antibody and targets together are recognized as "foreign" by the host's cellular immune system. In other words the binding of the immunoglobulin of the present invention enhances the phagocytization of the amyloid fibrils.

25 "Amyloidosis", as used herein, is intended to refer to any condition that is characterized by the presence of amyloid material. Such material may be in the form of an amyloidoma or more disperse amyloid deposits or fibrils.

Pharmaceutical Compositions

30 The pharmaceutical compositions for therapeutic treatment according to the present invention are intended for parenteral, oral or local administration. Preferably, the

pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. As the blood brain barrier is impermeable to IgG (see U. Bickel *et al.*, 1994 *Bioconjug. Chem.* 5: 119-25), delivery of antibodies to overcome the blood-brain barrier (BBB) may be achieved through

5 liposomal or micellar delivery of the antibody to the desired site. Alternatively, the agents of this invention can be delivered directly into the cerebrospinal fluid (see for example L.C. Walker *et al.*, 1994 *J. Neuropathol. Exp. Neurol.* 53: 377-83). For other delivery mechanisms, refer to P.M. Friden, 1996 U.S. Patent No. 5,527,527 and W.M. Pardridge, 1991 U.S. Patent No. 5,004,697. All of the above documents are incorporated

10 herein by reference.

Thus, the invention provides compositions for parenteral administration which comprise a solution of the anti-amyloid immunoglobulin polypeptide dissolved or suspended in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3%

15 glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required

20 to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of anti-amyloid immunoglobulin polypeptides of the invention

25 in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 1%, usually at or at least about 10-15% to as much as 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in an accordance with the particular mode of administration selected.

Without undue experimentation, one of ordinary skill in the art could determine

30 the quantity of immunoglobulin polypeptides that would be effective in adequately opsonizing an amyloidoma. Amounts effective for this use will depend on, *e.g.*, the

nature of the anti-amyloid immunoglobulin polypeptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician. A typical single dose of 0.5 mg/kg could generally be used. It must be kept in mind that the 5 anti-amyloid immunoglobulin polypeptide and peptide compositions derived therefrom may be employed in serious disease states, that is, life-threatening or potentially life-threatening situations. In such cases it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions. Thus, human anti-amyloid monoclonal antibodies or substantially human anti-amyloid receptor 10 monoclonal antibodies of the invention are most preferred under these circumstances.

Treatment of humans with amyloidosis according to the present invention could also be applied to animals susceptible to amyloidosis, such as cows or chickens. Thus, references to human patients herein apply also to non-human patients.

The immunoglobulin polypeptides, as defined herein, are preferably anti-amyloid 15 mAbs directed toward an amyloidoma or components or precursors thereof. The mAbs can be raised against IgLC variable region domains or, preferably, against the IgLC subsets κ1, κ4, λ8, or combinations thereof. The administration to humans of immunoglobulin polypeptides that are substantially non-human may elicit anti-antibody responses. Thus, it may be desirable to prepare anti-IgLC immunoglobulin polypeptides 20 of the present invention which are substantially human. By "substantially human" is meant an antibody or binding fragment thereof comprised of amino acid sequences which are at least about 50% human in origin, at least 70 to 80% more preferred, and about 95-99% or more human most preferred, particularly for repeated administrations over a prolonged period as may be necessary to treat established cases of amyloidosis. 25 As used herein, human antibody is meant to include antibodies of entirely human origin as well as those which are substantially human, unless the context indicates otherwise.

Monoclonal antibodies can also be raised against synthetic amyloid fibrils. Recombinant light chain, variable region peptides are isolated and purified *in vitro* using standard techniques. Synthetic fibrils are then prepared from the peptides using 30 techniques such as those described by Wall et al., "*In vitro* Immunoglobulin Light Chain Fibrillogenesis," METHODS IN ENZYMOLOGY, Vol. 309 (In Press). Antibodies are

then raised against the synthetic fibrils using standard immunization techniques, typically in mice or rabbits. Monoclonal cell lines secreting anti-fibril antibodies are produced using standard hybridoma techniques.

The anti-amyloid immunoglobulin polypeptides of the invention may be prepared 5 by any of a number of well-known techniques. For instance, they may be prepared by immunizing an animal with purified or partially purified human amyloid. The animals immunized can be any one of a variety of species which are capable of immunologically recognizing epitopes characteristic of the human type amyloid extracellular domain, such as murine, lagomorph, equine, etc.

10 Monoclonal antibodies of the invention may be prepared by immortalizing cells comprising nucleic acid sequences which encode immunoglobulin polypeptides or portions thereof that bind specifically to antigenic determinants characteristic of the extracellular domain of the human type amyloid. The immortalization process can be carried out by hybridoma fusion techniques, by viral transformation of antibody-
15 producing lymphocytes, recombinant DNA techniques, or by techniques that combine cell fusion, viral transformation and/or recombinant DNA methodologies. Immunogens to raise the monoclonal antibodies include synthetic amyloid fibrils as described, for example by, A. Lomakin *et al.*, 1997 Proc. Nat'l Acad. Sci. USA **94**: 7942-7, which is incorporated herein by reference.

20 As the generation of human anti-amyloid monoclonal antibodies may be difficult with conventional immortalization techniques, it may be desirable to first make non-human antibodies and then transfer via recombinant DNA techniques the antigen binding regions of the non-human antibodies, *e.g.*, the Fab, complementarity determining regions (CDRs) or hypervariable regions, to human constant regions (Fc) or framework regions
25 as appropriate to produce substantially human molecules. Such methods are generally known in the art and are described in, for example, U.S. 4,816,397, PCT publication WO 90/07861, and EP publications 173494 and 239400, wherein each is incorporated herein by reference. However, completely human antibodies can be produced in transgenic animals. The desired human immunoglobulin genes or gene segments can be isolated,
30 for example by PCR from human B cells, the DNA cloned into appropriate vectors for expression in eukaryotic cells and the cloned DNA introduced into animals to produce

transgenics. Animals suitable for the production of transgenics expressing human immunoglobulin include mice, rats, rabbits and pigs with rodents of transgenics that express human immunoglobulins should preferably have one or more of their endogenous immunoglobulin loci inactivated or "knocked-out" to facilitate identification
5 and isolation of the human antibodies (See e.g., Lonberg, et al. *Nature* 368:856-859 (1994)).

The resulting chimeric antibodies or chimeric immunoglobulin polypeptides that bind to human amyloid are also within the scope of the present invention. A typical therapeutic chimeric antibody would be a hybrid protein consisting of the variable (V) or
10 antigen-binding domain from a mouse immunoglobulin specific for a human amyloid antigenic determinant, and the constant (C) or effector domain from a human immunoglobulin, although domains from other mammalian species may be used for both variable and constant domains. As used herein, the term "chimeric antibody" also refers to antibodies coded for by immunoglobulin genes in which only the CDRs are
15 transferred from the immunoglobulin that specifically recognizes the antigenic determinants, the remainder of the immunoglobulin gene being derived from a human (or other mammalian, as desired) immunoglobulin gene. As discussed before, this type of chimeric antibody is referred to as a "humanized" (in the case of a human immunoglobulin gene being used) antibody. Also considered are recombinant human
20 antibodies that do not contain sequences of another species.

The hypervariable regions of the variable domains of the anti-amyloid immunoglobulin polypeptides comprise a related aspect of the invention. The hypervariable regions, or CDRs, in conjunction with the framework regions (those portions of immunoglobulin light and heavy chain variable regions that are relatively
25 conserved among different immunoglobulins in a single species), enable the anti-amyloid immunoglobulin polypeptides to recognize and thus bind to human amyloid. The hypervariable regions can be cloned and sequenced. Once identified, these regions that confer specific recognition of human amyloid can then be cloned into a vector for expression in a host as part of another immunoglobulin molecule or as a fusion protein,
30 e.g., a carrier molecule which functions to enhance immunogenicity of the cloned idiotype.

The anti-amyloid immunoglobulin polypeptides of the invention will generally be used intact, or as immunogenic fragments, such Fv, Fab, F(ab')₂ fragments. The fragments may be obtained from antibodies by conventional techniques, such as by proteolytic digestion of the antibody using, e.g., pepsin, papain or other proteolytic enzymes, or by recombinant DNA techniques in which a gene or portion thereof encoding the desired fragment is cloned or synthesized, and expressed in a variety of hosts.

Those skilled in the art will realize that "anti-idiotypic" antibodies can be produced by using a specific immunoglobulin as an immunogen in accordance with standard techniques. For example, infection or immunization with an amyloid fibril or fragment thereof, induces a neutralizing immunoglobulin, which has on its Fab variable region combining site an image of the amyloid that is unique to that particular immunoglobulin, i.e., an idioype. Immunization with such an anti-amyloid immunoglobulin induces an anti-idiotype antibody, which has a conformation at its combining site that mimics the structure of the original amyloid antigen. These anti-idiotype antibodies may therefore be used instead of the amyloid antigen. See, for example, Nisonoff (1991) J. Immunol. 147:2429-2438, which is incorporated herein by reference.

The following working examples specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art.

Example 1 Unassisted Resolution of Human IgLC Amyloid in Murine Host

Human IgLC amyloid was extracted and purified from infected organs obtained during an autopsy. The first experiments involved transplanting 50-200 mg of this amyloid material into a Balb/c mouse. The amyloid mass, or "amyloidoma," was prepared in sterile PBS by serial sonication and grinding steps in order to produce a fine suspension of amyloid fibrils complete with the accessory molecules found *in vivo*. This procedure was performed to allow the amyloid to be injected into the mice through a wide-gauge hypodermic needle.

The amyloid material, equivalent to 10% of the body weight of the animal, was injected into mice (under anesthetic) between the scapula, which resulted in a large mass being visible (see Figure 1A). The mouse required 15-18 days to achieve the complete removal of the amyloidoma (see Figure 1B), after which the animal appeared healthy and 5 lived a normal life span. The removal of the amyloidoma was determined subjectively by the experimenter; by simply palpating the injection site, an amyloidoma, like a hard pea, can be easily felt under the skin.

Example 2 Involvement of Both Antibody-Mediated and Cellular Immunity in the
10 Removal of Amyloidomas

The involvement of anti-amyloid antibodies in the removal of amyloidomas was shown by screening serum from a mouse previously injected with amyloid material against a sample of the injected material. This was done by Western blot analysis using suitable dilutions of the mouse serum as the primary antibody. It was shown that there 15 were antibodies to every component of the amyloid matrix, *i.e.*, every band on the gel was stained by the mouse serum, even at a 10,000-fold serum dilution (data not shown).

The involvement of a cellular component was demonstrated by *in vitro* neutrophil binding assays (see Figures 2A and 2B) and by using knockout-mutant mouse strains (data not shown). Figures 2A and 2B show human neutrophils adhering to human 20 amyloid after the amyloid was treated with mouse anti-human IgLC mAbs. This shows that the mouse mAb can bind to human amyloid as well as attract human neutrophils.

Studies of knockout-mutant mouse strains further support a finding of antibody involvement in amyloid removal. First, *scid/scid* mice, which lack B and T lymphocytes, were unable to remove an injected amyloidoma even after three months (data not shown). 25 Second, CD18 knockout animals were unable to remove the amyloidoma as rapidly as normal animals. CD18 knockout animals are 97% deficient in CD18, a cell surface integrin found on granulocyte/macrophage lineages. Although these cells cannot leave the circulation, the animals are B and T cell competent and can therefore mount an antibody response. Third, nude mice, which have no white blood cells, were unable to 30 remove the amyloidoma.

Furthermore, amyloid that had been incubated with amyloid-reactive serum from another mouse, when implanted into the second mouse, was removed within 4 days. In this experiment a Balb/c mouse was injected with 50 mg HIG amyloid and left for 1 week, after which it was bled by tail-vein clipping. The blood was spun down at 1500

5 rpm and the cells removed by aspiration. The plasma was stored at 4 °C until used.

Another preparation of HIG amyloid (100 mg) was prepared by suspending in sterile PBS to which was added 1 ml of plasma from the previous mouse. This preparation was then injected into a second mouse (Balb/c) and the amyloid was removed in 4 days.

Thus, it was concluded that the process could be sped up by opsonizing the material prior
10 to injection.

Example 3 ELISA Screening of IgLC Subsets

A systematic study was performed using ELISA techniques to screen a large number of human extracted amyloid samples using mAbs raised against the IgLC subsets
15 ($\lambda_1, \lambda_2, \lambda_3, \lambda_4, \lambda_5, \lambda_6, \kappa_1, \kappa_2, \kappa_3, \kappa_4$, free κ and λ and total κ and λ). Interestingly, it was found that more often than not, the amyloids tested positive with mAbs specific for their own subtype, the total κ or λ antibodies and a $\kappa_1(57-18H12)$, $\kappa_4(11-1F4)$ and $\lambda_8(31-8C7)$ mAb. These latter three reagents were found to react in a non-subgroup specific manner, *i.e.*, κ_1 reacted with amyloids comprised of IgLCs other than κ_1 ; and
20 the other two mAbs exhibit the same quality. This shows that the epitope recognized by these antibodies may be a general feature of amyloid fibrils, indicating the possibility of a shared amyloid epitope that can be targeted.

Example 4 Immunochemical Staining

25 Tissue samples from amyloid patients were stained using standard immunochemical techniques and a similar binding phenomenon was observed. Figures 3A-3D show that anti- κ_1 binds to the κ_1 amyloid and, surprisingly, that the anti- κ_4 reacts with the κ_1 amyloid, suggesting an amyloid epitope that these antibodies may recognize. Additionally, the anti- κ_4 reacts with λ -containing amyloid (not illustrated).
30 This is an example of cross-isotype reactivity. However, the results from the ELISA and the immunohistochemistry were not always consistent. This is likely due to the inherent

difference in what you are looking at, *i.e.*, ELISA is a liquid phase binding assay using extracted purified amyloid, whereas immunohistochemistry is performed on fixed tissue sections on a slide.

Samples of hybridoma cells that secrete anti- κ 1 (57-18-H12 (ATCC Acc. No.____)), anti- κ 4 (11-1F4 (ATCC Acc. No.____)) and anti- κ 8 (31-8c7 (ATCC Acc. No.____)) monoclonal antibodies were deposited with the American Type Culture Collection (ATCC) on May 21, 1999 in compliance with the Budapest Treaty.

Example 5 In Vivo Studies of Anti-IgLC Subgroups

10 0.1 mg of one of three antibodies -- κ 1, κ 4, or λ 8, identified above -- was injected into the thigh of a mouse into which amyloid had been introduced in the form of an amyloidoma as described above. The κ 1 and κ 4 reagents resulted in the complete removal by the host of most amyloid fibril species tested within 7 days (as little as 4 days for certain sources of amyloid). Figure 4 shows fluoresceinated κ 4 mAb binding to

15 human amyloid.

The λ 8 reagent, which is reactive in certain instances in both *in vitro* studies (above), increased the resolution of amyloidomas by up to about 10% in *in vivo* experiments.

20 *Example 6 In Vivo Studies of Anti-IgLC Subgroups*

Human amyloid was isolated from a patient with inflammation-associated, AA-amyloid and prepared for injection into Balb/C mice by repeated sonication and grinding in order to permit its injection into the mouse (see Example 1). Immediately after the injection of 100 mg of human AA-amyloid extract, the mice were treated with 100 μ g of

25 κ 4 mAb, anti-AA mAb, no mAb and non-specific control mAb (anti-free κ). Complete resolution of the material was observed with 48 hours in the animals that had been treated with the κ 4 and anti-AA mAbs. In contrast, the control animals had a large mass of amyloid remaining at the site of injection.

30 *Example 7 Production of Specific Anti-Amyloid Fibril mAbs*

Synthetic amyloid fibrils were prepared *in vitro* and used as an immunogen in mice to produce a first generation of anti-amyloid fibril mAbs. Briefly, recombinant $\lambda 6$ -light chain, variable region peptides were produced, isolated and purified using a bacterial expression system and standard protein purification techniques. Synthetic 5 fibrils were prepared from these peptides by extended periods of agitation in solution as described, for example, in Wall et al., "In vitro Immunoglobulin Light Chain Fibrillogenesis," METHODS IN ENZYMOLOGY, Vol. 309 (In Press), which is incorporated herein by reference in its entirety. Fibrils were concentrated by centrifugation at 17,000 \times g for 20 minutes at room temperature.

10 The concentrated fibrils were then used to immunize Balb/c mice over a period of several weeks. Monoclonal cell lines secreting anti-fibril antibodies were produced using standard hybridoma techniques. The resultant antibodies have demonstrable anti-fibril activity based upon ELISA assays, described in Example 3. These antibodies reacted with 99% of all human IgLC amyloid extracts tested to date irrespective of the 15 nature of the isotype or subgroup of the precursor protein when tested by ELISA. Similarly, the antibodies reacted in an ELISA format with isolated murine AA-amyloid and synthetic fibrils composed of a peptide derived from the Alzheimer's protein A β [A β (25-35)].

It should be understood that the foregoing discussion and examples merely 20 present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All references, articles and patents identified above are herein incorporated by reference in their entirety.

What is claimed is:

1. A method of treating a patient having an amyloid deposition disease comprising the step of administering to the patient
 - 5 a) a therapeutically effective dose of at least one immunoglobulin polypeptide or a fragments thereof, wherein the immunoglobulin polypeptide or fragment thereof binds to an amyloid fibril; and
 - b) a pharmaceutically acceptable carrier.
 - 10 2. The method of claim 1, wherein the immunoglobulin polypeptide or fragment thereof is raised against an immunoglobulin light-chain.
 3. The method of claim 1, wherein binding of the immunoglobulin polypeptide or fragment thereof opsonizes the amyloid fibril.
 - 15 4. The method of claim 1, wherein the immunoglobulin polypeptide or fragment thereof is a monoclonal antibody.
 5. The method of claim 4, wherein the monoclonal antibody is a humanized antibody.
 - 20 6. The method of claim 4, wherein the monoclonal antibody is a chimeric antibody.
 - 25 7. The method of claim 6, wherein the chimeric antibody is a humanized antibody.
 8. The method of claim 4, wherein the antibody is a labeled antibody.
 - 30 9. The method of claim 4, wherein the monoclonal antibody is selected from the group consisting of κ1 (57-18H12), κ4 (11-1F4), λ8 (31-8C7), and combinations thereof.

10. An immunoglobulin polypeptide or fragment thereof that binds to an amyloid fibril and is effective to enhance the cellular immune response of a patient to remove disease-associated amyloid fibril deposits.

5 11. The immunoglobulin polypeptide or fragment thereof of claim 10, wherein the immunoglobulin polypeptide or fragment thereof is a monoclonal antibody or fragment thereof.

12. The immunoglobulin or fragment thereof of claim 11, wherein the
10 monoclonal antibody is a humanized antibody.

13. The immunoglobulin polypeptide or fragment thereof of claim 11, wherein the monoclonal antibody is a chimeric antibody.

15 14. The immunoglobulin polypeptide or fragment thereof of claim 13, wherein the chimeric antibody is a humanized antibody.

15. The immunoglobulin polypeptide or fragment thereof of claim 11, wherein the antibody is a labeled antibody.

20 16. The immunoglobulin polypeptide or fragment thereof of claim 11, wherein the monoclonal antibody is selected from the group consisting of κ1 (57-18H12), κ4 (11-1F4), λ8 (31-8C7), and combinations thereof.

25 17. The monoclonal antibody or fragment thereof of claim 16, wherein the monoclonal antibody is a humanized antibody.

18. The immunoglobulin polypeptide or fragment thereof of claim 10, wherein the immunoglobulin polypeptide or fragment thereof has been raised against synthetic
30 amyloid fibrils.

19. A pharmaceutical composition comprising the immunoglobulin peptide or fragment thereof of any of claims 10-17.
20. A nucleic acid molecule which encodes a polypeptide comprising at least a 5 hypervariable region of the immunoglobulin polypeptide of any of claims 10-17.
21. A host cell comprising a nucleic acid molecule of claim 20.
22. A method of producing an immunoglobulin polypeptide comprising the step 10 of culturing the host cell of claim 21.

1/4

FIG. 1A



Day 1

FIG. 1B



Day 14

2/4

FIG. 2A

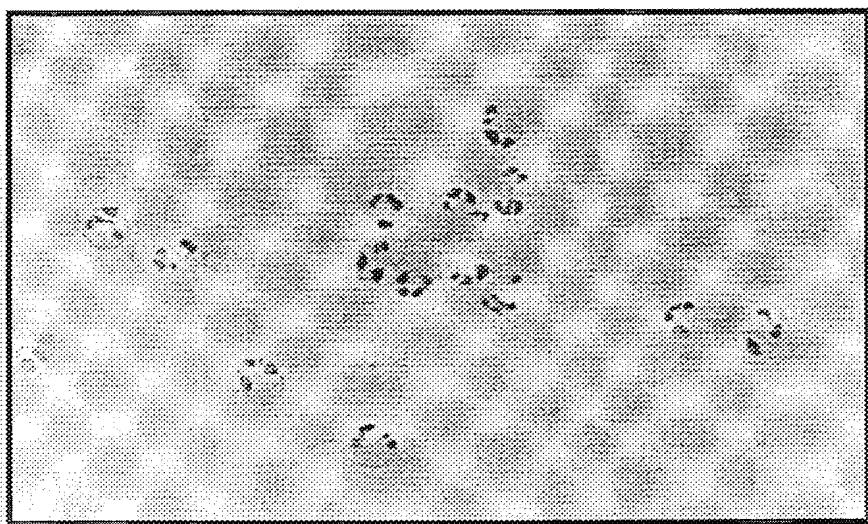


FIG. 2B

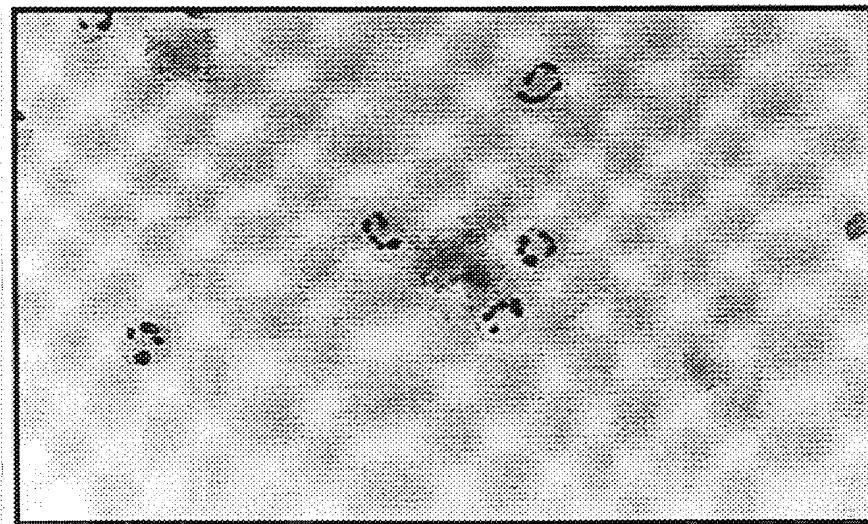


FIG. 3A

Her



CR

FIG. 3B*FIG. 3C**FIG. 3D*

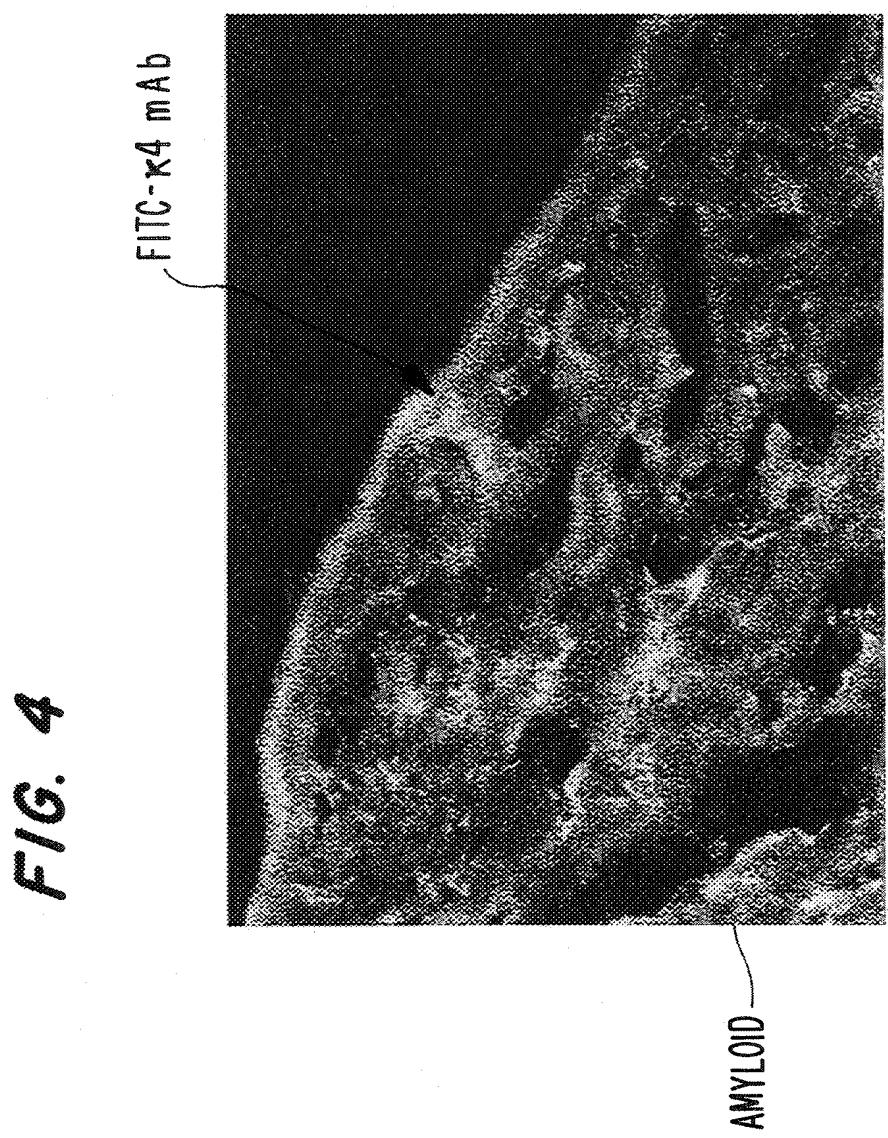
3/4

anti-kI (57-18-H12)

anti-kIV (11-1F4)

anti-lambda VIII (31-8C7)

4/4



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/11200

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 16/18, A61K 39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9625435 A1 (BAYER CORPORATION), 22 August 1996 (22.08.96), See page 1, lines 4-7 and lines 19-23; page 6, lines 11-22; page 7, lines 21-24; page 12, lines 19-20	1,4,8,10,11, 18-22
A	---	2,3,5-7,9, 12-17

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

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31 August 1999

Date of mailing of the international search report

07.10.1999

Name and mailing address of the International Searching Authority
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/11200

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Bioconjugate Chem., Volume 5, 1994, Ulrich Bickel et al, "Development and in Vitro Characterization of a Cationized Monoclonal Antibody against BetaA4 Protein: A Potential Probe for Alzheimer's Disease", page 119 - page 125, see abstract and page 124, lines 6-14 (right column)	10-12,15, 20-22
A	-- --	1-9,13,14, 16-19
A	WO 8901343 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 23 February 1989 (23.02.89)	1-22
A	Journal of Neuropathology and Experimental Neurology, Volume 53, No 4, July 1994, Lary C. Walker et al, "Labeling of Cerebral Amyloid in Vivo with a Monoclonal Antibody", page 377 - page 383, See abstract	1-22
	-- -----	

INTERNATIONAL SEARCH REPORT

Int'l. application No.

PCT/US 99/11200

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-9 relate to a method of treatment of the human or animal body by surgery or therapy/diagnostic methods practised on the human or animal body/Rule 39.1(iv), the search has been carried out and based on the alleged effects of the compounds/compositions.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

S/ 35950

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/08/99

International application No.

PCT/US 99/11200

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9625435 A1	22/08/96	CA 2212887 A		22/08/96
		EP 0809656 A		03/12/97
		JP 10509736 T		22/09/98
		US 5679531 A		21/10/97
		US 5693753 A		02/12/97
		US 5786180 A		28/07/98
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WO 8901343 A1	23/02/89	AT 123227 T		15/06/95
		AU 628299 B		17/09/92
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		CA 1309340 A		27/10/92
		DE 3853923 D,T		16/11/95
		EP 0557270 A,B		01/09/93
		JP 3500644 T		14/02/91
		US 5004697 A		02/04/91
<hr/>				



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ :	A2	(11) International Publication Number:	WO 00/20027
A61K 39/00		(43) International Publication Date:	13 April 2000 (13.04.00)
(21) International Application Number:	PCT/DK99/00525	KARLSSON, Gunilla [SE/DK]; Collinsgade 6A, 3. tv., DK-2100 København ø (DK).	
(22) International Filing Date:	5 October 1999 (05.10.99)	(74) Agent:	PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annae Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).
(30) Priority Data:	PA 1998 01261 60/105,011	5 October 1998 (05.10.98) 20 October 1998 (20.10.98)	US
(71) Applicant (for all designated States except US):	M & E BIOTECH A/S [DK/DK]; Kogle Allé 6, DK-2970 Hørsholm (DK).	(81) Designated States:	AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(72) Inventors; and		Published	Without international search report and to be republished upon receipt of that report.
(75) Inventors/Applicants (for US only):	STEINAA, Lucilla [DK/DK]; Viktoriagade 28, 3. th., DK-1655 København V (DK). MOURITSEN, Søren [DK/DK]; Lindevangsvej 24, DK-3460 Birkerød (DK). NIELSEN, Klaus, Gregorius [DK/DK]; Lykkesborg Allé 15, DK-2860 Søborg (DK). HAANING, Jesper [DK/DK]; Biskop Svanes Vej 2E, DK-3460 Birkerød (DK). LEACH, Dana [US/DK]; Ved Store Dyrehave 44, DK-3400 Hillerød (DK). DALUM, Iben [DK/DK]; Olgasvej 13, DK-2970 Hørsholm (DK). GAUTAM, Anand [GB/DK]; Ved Store Dyrehave 22, st.tv., DK-3400 Hillerød (DK). BIRK, Peter [DK/DK]; Rudolph Berghsgade 5, DK-2100 København ø (DK).		
(54) Title:	NOVEL METHODS FOR THERAPEUTIC VACCINATION		
(57) Abstract	<p>A method is disclosed for inducing cell-mediated immunity against cellular antigens. More specifically, the invention provides for a method for inducing cytotoxic T-lymphocyte immunity against weak antigens, notably self-proteins. The method entails that antigen presenting cells are induced to present at least one CTL epitope of the weak antigen and at the same time presenting at least one foreign T-helper lymphocyte epitope. In a preferred embodiment, the antigen is a cancer specific antigen, e.g. PSM, Her2, or FGF8b. The method can be exercised by using traditional polypeptide vaccination, but also by using live attenuated vaccines or nucleic acid vaccination. The invention furthermore provides immunogenic analogues of PSM, Her2 and FGF8b, as well as nucleic acid molecules encoding these analogues. Also vectors and transformed cells are disclosed. The invention also provides for a method for identification of immunogenic analogues of weak or non-immunogenic antigens.</p>		

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NOVEL METHODS FOR THERAPEUTIC VACCINATION

FIELD OF THE INVENTION

The present invention relates to novel methods for combatting diseases, such as cancers, which are characterized by the presence of cell-associated gene expression products which are non-immunogenic or poorly immunogenic. In particular, the present invention relates to methods for inducing an immune response conducted by cytotoxic T-lymphocytes (CTLs), whereby cells carrying epitopes from the gene expression products are attacked and killed by the CTLs. The invention also relates to a method of preparing immunogenic, modified polypeptide antigens which are derived from weakly immunogenic antigens.

The invention further relates to a series of applications of Applicants' AutoVac technology (which is the subject of WO 95/05849) within the field of therapeutic vaccination against cancer.

BACKGROUND OF THE INVENTION

The idea of vaccinating against cancer has been around for more than hundred years and has enjoyed recurrent bursts of activity, particularly since the turn of this century.

However, during the past 10 years the understanding of the fundamental molecular mechanisms of the immune response has improved considerably. Among the most important milestones achieved during this period has been the discovery of the still growing list of cytokines and growth factors, the understanding of the mechanisms of interaction between T and B cells as well as the establishment of the cellular antigen processing pathways including the role and structure of the MHC class I and II molecules in antigen presentation. Impor-

tant discoveries with regard to cancer immunology - although still not fully understood - were also the elucidation of the mechanisms underlying induction of immunological tolerance in a host. All this research has led to a huge amount of efforts 5 in order to develop new treatments for human cancer.

Depending on how tumour immunity is acquired by the patient, immunotherapy regimens can be categorised as either passive or active. In passive immunotherapy regimens the patient passively receives immune components such as cytokines, antibodies, 10 cytotoxic T-cells, or lymphocyte activated killer (LAK) cells. In contrast, active specific immunotherapy protocols encompass actively inducing tumour immunity by vaccination with the tumour cell or its antigenic components. This latter form of treatment is preferred because the immunity is prolonged.

15 Passive and active cancer vaccines have focussed on inducing either humoral or cellular immune responses. For active vaccines it is well established that induction of CD4 positive T helper cells is necessary in order to secondarily induce either antibodies or cytotoxic CD8 positive T cells.

20 *Passive vaccination with antibodies*

Since the discovery of the monoclonal antibody technology in the mid-seventies, a large number of therapeutic monoclonal antibodies directed against tumour specific or tumour associated antigens has been developed. Monoclonal antibody therapy, 25 however, gives rise to several serious problems:

- Injection of these foreign substances induces an immune response in the patient towards the injected antibodies, which may lead to less efficient treatment as well as to serious allergic side-effects in the patients.

- Monoclonal antibodies usually must be administered in large amounts. This is a problem, since the production costs of monoclonal antibodies are huge.

5

- Monoclonal antibodies must be administered via the parenteral route and due to the relatively large amounts needed, the patients frequently must be hospitalised during the treatment.

10

- Injections of monoclonal antibodies must be repeated at rather short intervals (weeks) in order to maintain therapeutic effect.

15

- Monoclonal antibodies are usually not able to activate secondary effector systems of the immune system such as complement, NK-cells or macrophage killing of tumour cells.

20

The latter disadvantage is of particular importance in cancer therapy and may be an important reason why monoclonal antibody therapy of cancer in several cases has not been particularly successful. The so-called humanised monoclonal antibodies now used by many companies are less immunogenic, but unfortunately they are even less capable of activating the secondary immune effector systems. Furthermore, examples of secondary out-growth of tumours lacking the original tumour antigen have been observed, since these antibodies do not induce "innocent bystander" effects on tumour cells not carrying the tumour 25 antigen.

The poor effector capability of the monoclonal antibodies has led to the development of monoclonal antibodies chemically conjugated to different toxins and radioisotopes. Pharmacia Upjohn AB has e.g. developed a conjugate between a monoclonal 30 tumour specific antibody and the *Staphylococcus aureus* toxin A

with the purpose of activating T cells in the tumour. Medarex Inc. has developed bispecific monoclonal antibodies containing a tumour specific Fab fragment as well as an Fc-receptor specific antibody fragment with the purpose of activating 5 macrophage killing of tumour cells. Both constructs are more effective than the monoclonal antibody alone, but they are also more expensive and immunogenic. Antibodies conjugated to radioisotopes are also expensive as well as immunogenic and other general toxic side-effects are observed.

10 The appearance of the monoclonal antibody technology was a major step forward which enabled the production of well-defined, high-affinity binding molecules. However, being monoclonal these antibodies only react with a single type of epitope on a tumour antigen. This is the major reason why they 15 usually are not able to activate the complement system or binding to the Fc-receptors of NK-cells and macrophages. These very powerful effector systems usually require the co-localisation of multiple Fc antibody fragments protruding from the antigen.

20 Other researchers have therefore attempted to use two monoclonal antibodies in combination and this has led to an improved effect. It therefore seems very reasonable instead to attack tumour cells with highly specific polyclonal antibodies di- rected against a tumour specific, or against (over-expressed) 25 tumour associated antigens or growth factor receptors. Such antibodies would be fully capable of activating the secondary effector systems mentioned above. Furthermore, it is likely that the local inflammatory reaction induced by these effector systems could lead to secondary effects on "innocent bystander" cells not expressing the tumour antigen in question 30 as well as to activation of tumour specific TIL's (tumour infiltrating lymphocytes) in the tumour tissue. Such effects

have been observed by Medarex Inc. using their bi-specific monoclonal antibody conjugates.

Since the discovery of the monoclonal antibody technology the potential use of polyclonal antibodies for cancer therapy has 5 not been explored very much (except for the antigens described below). One major reason is that well-defined tumour specific or tumour associated surface antigens only have been characterised within the recent years, but - more importantly - many of these have turned out to be self-antigens and therefore 10 non-immunogenic. Accordingly, xenogenic polyclonal antibodies would necessarily have been used to study the effects. However, such antibodies induce a vigorous immune response towards the injected foreign polyclonal antibodies which rapidly eliminate the therapeutic effects.

15 *Active vaccination to induce antibodies*

Recent attempts to induce therapeutic polyclonal autoantibodies in cancer patients by active vaccination have been successful. Vaccines against membrane bound carbohydrate self-antigens (such as the O-linked aberrantly expressed Tn and 20 sTn-antigens and the ganglioside liposaccharides GM2 and GD3) have been developed. These small carbohydrate structures are, however, very poor antigens so conjugates of these molecules with carrier molecules such as keyhole limpet haemocyanin (KLH) or sheep mucins (containing Tn- and sTn) must be used. 25 In melanoma patients the induction of anti-GM2 antibodies were associated with a prolonged disease-free interval and overall survival after a minimum follow-up of fifty-one months. Also randomised phase II studies have been conducted on breast cancer patients using a conjugate of sTn and KLH in the DETOX-30 B adjuvant (BIOMIRRA Inc.) showing that sTn immune patients had a significantly longer median survival compared to controls. Another example of the active induction of polyclonal antibo-

dies in cancer is the use of idiotype specific vaccination against B-cell lymphomas, which - although it has been promising - is limited to this cancer type only.

Finally, the US company Aphton Inc. has developed active
5 conjugate vaccines against gonadotropin releasing hormone (GnRH) and gastrin. It has been demonstrated, that this vaccine is capable of controlling the biological activity of these hormones, which also can function as autocrine growth factors for certain tumour cells. Successful phase II clinical
10 trials have been conducted on gastrointestinal cancer patients and phase III clinical trials are underway.

Cytotoxic T-cells

It has been clearly demonstrated by several groups that tumour specific cytotoxic T cells (CTL's) are present in many tumours. These CTL's are termed tumour infiltrating lymphocytes (TIL's). However, these cells are somehow rendered non-responsive or anergic by several different possible mechanisms including secretion of immunosuppressive cytokines by the tumour cells, lack of co-stimulatory signals, down regulation
20 of MHC class I molecules etc.

There has been many attempts to isolate the tumour specific HLA class I bound peptides recognised by TILs, and in some cases it has also been successful (e.g. peptides from the melanoma associated antigens). Such peptides have been used to
25 induce a tumour specific immune response in the host, but the practical use of tumour specific peptides in vaccines is restricted to a limited segment of the population due to the narrow HLA class I binding specificity of the peptides. Furthermore, it is usually relatively difficult to evoke a CTL
30 response *in vivo* using synthetic peptides due to the low

biological half-life of these substances as well as the difficulties with exogenous priming of MHC class I molecules.

Many other approaches have been attempted in order to evoke a tumour specific CTL response including the use of cytokines
5 (e.g. IL-2, IFN- γ , IL-6, IL-4, IL-10 or GM-CSF) or co- stimulatory molecules (B7) either in soluble form or expressed by the transfected tumour cell. Furthermore, immunisations with allogenic or autologous whole cells, or of tumour antigens prepared in specialised adjuvants designed to present the
10 antigen via the MHC class I antigen presentation route, or tumour antigens expressed in e.g. vaccinia vectors etc. have been used with varying success. Still the general belief among tumour immunologists is therefore that one of the best ways to eliminate tumours would be to induce a strong specific anti-
15 tumour CTL response.

Apart from the fact that these treatments usually are very expensive and difficult to reproduce, it has also turned out to be difficult to obtain a good immune response towards the tumour since many of the tumour associated antigens are true
20 self-proteins to which most T cells appear to be tolerant. Therefore, it seems necessary to induce a controlled cellular autoimmune condition in the patient.

OBJECT OF THE INVENTION

It is an object of the present invention to provide improved
25 methods and agents for inducing immune responses in host organisms against undesirable antigens, e.g. tumour antigens. It is a further object to provide a method for preparing polypeptide analogues of such undesirable antigens, analogues which are capable of inducing an effective immune response
30 against the undesired antigen.

SUMMARY OF THE INVENTION

Presentation of antigens has dogmatically been thought of as two discrete pathways, a class II exogenous and a class I endogenous pathway.

5 Briefly, a foreign protein from outside the cell or from the cell membrane is taken up by the APC as an endosome which fuses with an intracellular compartment which contains proteolytic enzymes and MHC class II molecules. Some of the produced peptides bind to class II, which then are transloca-
10 ted to the cell membrane.

The class I endogenous pathway is characterised by the predominant presentation of cytosolic proteins. This is believed to occur by proteasome mediated cleavage followed by transportation of the peptides into the endoplasmic reticulum (ER) via
15 TAP molecules located in the membrane of the ER. In ER the peptides bind to class I followed by transportation to the plasma membrane.

However, these 2 pathways are not fully distinct. For example it is known that dendritic cells and to some extend macro-
20 phages are capable of endocytosing (pinocytosing) extracellular proteins and subsequently present them in the context of MHC class I. It has also previously been demonstrated that using specialised administration routes, e.g. by coupling to iron oxide beads, exogenous antigens are capable of entering
25 the Class I pathway (Rock, 1996). This mechanism seems central, because of the importance of a concomitant expression of both class I and class II on the same APC to elicit a three cell type cluster. This three cell type cluster of interaction has been proposed by Mitchison (1987) and later by other
30 authors. They showed the importance of concomitant presenta-

tion of class I and class II epitopes on the same APC. According to the recently described mechanism for CTL activation (cf. Lanzavecchia, 1998, Nature **393**: 413, Matzinger, 1999, Nature Med. **5**: 616, Ridge et al., 1998, Nature **393**: 474, Bennett et al., 1998, Nature **393**: 478, Schoenberger et al., 1998, Nature **393**: 480, Ossendrop et al., 1998, J. Exp. Med **187**: 693, and Mackey et al., 1998, J. Immunol **161**: 2094), professional APCs presenting antigen on MHC class II are recognized by T helper cells. This results in an activation of the APC (mediated by interaction by CD40L on the T helper cell and CD40 on the APC). This enables the APC to directly stimulate CTLs which are thereby activated. Cf. also Fig. 2.

It has previously been demonstrated that insertion of a foreign MHC class II restricted T helper cell epitope into a self-antigen results in the provision of an antigen capable of inducing strong cross-reactive antibody responses directed against the non-modified self-antigen (cf. applicant's WO 95/05849). It was shown that the autoantibody induction is caused by specific T cell help induced by the inserted foreign epitope.

However, we have come to the conclusion that modified self-antigens - with the aid of appropriate adjuvants - ought to be capable of also inducing strong CTL responses against MHC class I restricted self-epitopes and hence the technology described in WO 95/05849 can be adapted to also provide vaccination against intracellular and other cell-associated antigens which have epitopes presented in the context of MHC Class I.

The autovaccine technology described in WO 95/05849 has the effect that specific T cell help is provided to self-reactive B cells when a modified self-antigen is administered for uptake into the MHC class II antigen processing pathway (cf.

Fig. 1, and Dalum I *et al.*, 1996, J. Immunol. **157**: 4796-4804 as well as Dalum I *et al.*, 1999, Nature Biotechnol. **17**: 666-669). It was shown that potentially self-reactive B-lymphocytes recognizing self-proteins are physiologically present in 5 normal individuals. However, in order for these B-lymphocytes to be induced to actually produce antibodies reactive with the relevant self-proteins, assistance is needed from cytokine producing T-helper lymphocytes (T_H -cells or T_H -lymphocytes). Normally this help is not provided because T-lymphocytes in 10 general do not recognize T-cell epitopes derived from self-proteins when presented by antigen presenting cells (APCs). However, by providing an element of "foreignness" in a self-protein (*i.e.* by introducing an immunologically significant modification), T-cells recognizing the foreign element are 15 activated upon recognizing the foreign epitope on an APC (such as, initially, a mononuclear cell). Polyclonal B-lymphocytes (which present T-cell epitopes) capable of recognising self-epitopes on the modified self-protein also internalise the antigen and subsequently presents the foreign T-cell 20 epitope(s) thereof, and the activated T-lymphocytes subsequently provide cytokine help to these self-reactive polyclonal B-lymphocytes. Since the antibodies produced by these polyclonal B-lymphocytes are reactive with different epitopes on the modified polypeptide, including those which are also 25 present in the native polypeptide, an antibody cross-reactive with the non-modified self-protein is induced. In conclusion, the T-lymphocytes can be led to act as if the population of polyclonal B-lymphocytes have recognised an entirely foreign antigen, whereas in fact only the inserted epitope(s) is/are 30 foreign to the host. In this way, antibodies capable of cross-reacting with non-modified self-antigens are induced.

As mentioned above, CTL's also require specific T cell help, although the mechanism for this is still not clear.

We have based the present invention on our novel theory that the self-proteins containing foreign MHC class II epitopes, following exogenous uptake, can gain access into the MHC class I antigen processing pathway of e.g. macrophages and dendritic 5 cells. In this way a strong CTL response against subdominant epitopes in the self-protein could be induced. Alternatively, genes encoding modified tumour antigens could be administrated as nucleic acid vaccines eventually also leading to MHC class II as well as MHC class I mediated immune responses.

10 Tumour cells are very poor antigen presenting cells due to insufficient MHC class I expression, lack of co-stimulatory molecules or secretion of immunosuppressive cytokines etc. Using the autovaccine constructs and vaccination protocol mentioned above the modified tumour antigen could be presented 15 by MHC class I as well as by MHC class II molecules on professional antigen presenting cells. Co-presentation of subdominant self-epitopes on MHC class I and immunodominant foreign epitopes on MHC class II molecules would mediate a direct cytokine help from activated MHC class II restricted T-helper 20 cells to MHC class I restricted CTLs (Fig. 2). This will in our opinion lead to a specific break of the T cell autotolerance towards the tumour antigen and this is exactly what is desired in cancer immunotherapy.

In conclusion, a vaccine constructed using the technology 25 outlined above will induce a humoral autoantibody response with secondary activation of complement and antibody dependent cellular cytotoxicity (ADCC) activity. It is also expected that it will induce a cytotoxic T cell response directed against e.g. a tumour specific membrane antigen.

30 Hence, in the broadest and most general scope, the present invention relates to a method for inducing an immune response against a polypeptide antigen in an animal, including a human

being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, the method comprising effecting simultaneous presentation by antigen presenting cells (APCs) from the animal's immune system of an immunogenically effective amount of

- 1) at least one CTL epitope derived from the polypeptide antigen and/or at least one B-cell epitope derived from the cell-associated polypeptide antigen, and
- 2) at least one first T helper cell epitope (T_H epitope)

which is foreign to the animal.

In a more specific variant of the inventive method, the invention relates to a method for down-regulating a cell-associated polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, by inducing a specific cytotoxic T-lymphocyte (CTL) response against cells carrying the cell-associated polypeptide antigen on their surface or harbouring the cell-associated polypeptide antigen in their intracellular compartment, the method comprising effecting, in the animal,

simultaneous presentation by a suitable antigen presenting cell (APC) of

- 1) at least one CTL epitope derived from the cell-associated polypeptide antigen, and
- 2) at least one first T-helper lymphocyte (T_H) epitope which

is foreign to the animal.

Also, the novel strategy for preparing an immunogenic agent is part of the invention. This novel strategy encompasses the selection and production of analogues of weak cell-associated antigens, where the preservation of a substantial fraction of known and predicted CTL epitopes is aimed at while at the same time introducing at least one foreign T_H epitope.

Furthermore, the invention relates to certain specific immuno-
genic constructs based on known tumour-associated antigens as
well as to compositions containing these constructs.

Finally, the invention relates to nucleic acid fragments,
5 vectors, transformed cells and other tools useful in molecular
biological methods for the production of the analogues of the
tumour-associated antigens.

LEGENDS TO THE FIGURE

Fig. 1: The traditional AutoVac concept. A: Tolerodominant
10 self-epitopes presented on MHC class II on an antigen presenting
cell (APC) are ignored due to depletion in the T helper
cell (Th) repertoire (T helper cell indicated with dotted
lines). Inserted foreign immunodominant T cell epitopes pre-
sented on MHC class II activate T helper cells and B cells (B)
15 specific for native parts of the self-protein presenting
foreign immunodominant T cell epitopes on MHC class II are
activated by the cytokine help provided by the T helper cell.

Fig. 2: The AutoVac concept for inducing a CTL response.
Inserted foreign immunodominant T cell epitopes presented on
20 MHC class II activate T helper cells. CTL's recognising
subdominant self-epitopes presented on MHC class I are acti-
vated by the adjacent activated T helper cell.

Fig. 3: A schematic representation of the Her2 polypeptide
with indications of epitopic regions and N-glycosylation
25 sites. The 4 extracellular domains, the transmembrane (TM)
domain and the 2 intracellular domains are represented with
indications of sites with varying degrees of homology and
sites containing putative/determined CTL epitopes.

Fig. 4: A schematic representation of the human PSM polypeptide with indications of insertion regions for the P2 and P30 epitopes.

Fig. 5: The FGF genes and proteins. A: Exon-intron structure 5 of the human and mouse FGF8 genes. Below is illustrated the eight different splice forms (from Gemel 1996). B: Amino acid sequence of the different FGF8 isoforms. The polypeptide stretches unique to FGF8b, FGF8f, and FGF8e are indicated by bold and italic or underlined typefaces. FGF8a is the shortest 10 variant containing none of these highlighted sequences. The signal peptide is expected to be cleaved C-terminally to Ala22. The two cysteine residues found in mature FGF8 (all isoforms) are indicated by thick underlining. The two potential N-glycosylation sites of FGF8b are indicated by \tilde{N} . Numbering is according to FGF8b.
15

Fig. 6: Illustrations of the four different variants of FGF8b designed for autovaccination. Upper panel: Theoretical models of the insertion-points of the epitopes using the FGF2 crystal structure as template. Lower panel: Amino acid sequences of 20 the wild type FGF8b (WT) and the four variants F30N, F2I, F30I, and F2C. The signal peptide is marked with single underlining. The inserted peptides are marked with double underlining. The N-terminal sequence (MetAla) of all variants is due 25 to generation of a Kozak-sequence (Kozak 1991) for better translation in eukaryotic systems.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present specification and claims will be defined and explained in

detail in order to clarify the metes and bounds of the invention.

A "cell-associated polypeptide antigen" is in the present specification and claims intended to denote a polypeptide which is confined to a cell which is somehow related to a pathological process. Furthermore, the cell presents CTL epitopes of the polypeptide antigen bound to MHC Class I molecules on its surface. Cell-associated polypeptide antigens can therefore be truly intracellular antigens (and thereby unreachable for a humoral immune response) or antigens bound to the surface of the cells. The cell-associated antigen can be the product of the cell's own gene expression, of a intracellular parasite, of a virus, or of another cell. In the latter case the polypeptide antigen is subsequently associated with the cell which is involved in the pathological process.

The terms "T-lymphocyte" and "T-cell" will be used interchangeably for lymphocytes of thymic origin which are responsible for various cell mediated immune responses as well as for effector functions such as helper activity in the humoral immune response. Likewise, the terms "B-lymphocyte" and "B-cell" will be used interchangeably for antibody-producing lymphocytes.

An "antigen presenting cell" (APC) is a cell which presents epitopes to T-cells. Typical antigen-presenting cells are macrophages, dendritic cells and other phagocytizing and pinocytizing cells. It should be noted that B-cells also functions as APCs by presenting T_H epitopes bound to MCH class II molecules to T_H cells but when generally using the term APC in the present specification and claims it is intended to refer to the above-mentioned phagocytizing and pinocytizing cells.

"Helper T-lymphocytes" or "T_H cells" denotes CD4 positive T-cells which provide help to B-cells and cytotoxic T-cells via the recognition of T_H epitopes bound to MHC Class II molecules on antigen presenting cells.

5 The term "cytotoxic T-lymphocyte" (CTL) will be used for CD8 positive T-cells which require the assistance of T_H cells in order to become activated.

A "specific" immune response is in the present context intended to denote a polyclonal immune response directed predominantly against a molecule or a group of quasi-identical molecules or, alternatively, against cells which present CTL epitopes of the molecule or the group of quasi-identical molecules.

A "weak or non-immunogenic polypeptide antigen" is herein intended to denote polypeptides having the amino acid sequence of the weak cell-associated protein antigens derived from the animal in question (e.g. a human), but also polypeptides having the amino acid sequence identical to analogues of such proteins isolated from other species are embraced by the term. Also forms of the polypeptides having differing glycosylation patterns because of their production in heterologous systems (e.g. yeasts or other non-mammalian eukaryotic expression systems or even prokaryotic systems) are included within the boundaries of the term. It should, however, be noted that when using the term, it is intended that the polypeptide in question is normally non-immunogenic or only weakly immunogenic in its natural localisation in the animal to be treated.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Further-

more, the term is also intended to include proteins, i.e. functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently 5 linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups.

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleotides, derived directly from a naturally occurring amino acid 10 sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general intended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one single animal. However, the term also denotes a population of 15 such an animal species, since it is important that the individuals immunized according to the method of the invention all harbour substantially the same weak, cell-associated polypeptide antigen allowing for immunization of the animals with the same immunogen(s). If, for instance, genetic variants of 20 polypeptides exist in different human populations it may be necessary to use different immunogens in these different populations in order to be able to break the autotolerance towards the weak, cell-associated polypeptide antigen in each population.

25 By the term "down-regulation a cell-associated polypeptide antigen" is herein meant reduction in the living organism of the amount and/or activity of the antigen in question. The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in 30 the antigen by antibody binding is the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of the polypeptide by

scavenger cells (such as macrophages and other phagocytizing cells), and even more important, that cells carrying or harbouring the antigen are killed by CTLs in the animal.

The expression "effecting simultaneous presentation by a suitable APC" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner which results in the simultaneous presentation by APCs of the epitopes in question. As will appear from the disclosure below, such challenge of the immune system can be effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmacines" (i.e. a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the animal are confronted with APCs displaying the relevant epitopes in an immunologically effective manner.

The term "immunogenically effective amount" has its usual meaning in the art, i.e. an amount of an immunogen which is capable of inducing an immune response which significantly engages pathogenic agents which share immunological features with the immunogen.

When using the expression that the weak cell-associated polypeptide antigens have been subjected to a "modification" is herein meant a chemical modification of the polypeptide which constitutes the backbone of the polypeptide in question. Such a modification can e.g. be derivatization (e.g. alkylation) of certain amino acid residues in the amino acid sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise changes of the primary structure of the amino acid sequence.

When discussing "tolerance" and "autotolerance" is understood that since the polypeptides which are the targets of the present inventive method are self-proteins in the population to be vaccinated or proteins which do not result in induction 5 of an effective immune response, normal individuals in the population do not mount an immune response against the polypeptide. It cannot be excluded, though, that occasional individuals in an animal population might be able to produce antibodies against the native polypeptide antigen, e.g. as 10 part of a autoimmune disorder. At any rate, an animal will normally only be autotolerant towards its own polypeptide antigen, but it cannot be excluded that analogues derived from other animal species or from a population having a different phenotype would also be tolerated by said animal.

15 A "foreign T-cell epitope" is a peptide which is able to bind to an MHC molecule and stimulates T-cells in an animal species. Preferred foreign epitopes are "promiscuous" epitopes, i.e. epitopes which binds to a substantial fraction of MHC class II molecules in an animal species or population. Only a 20 very limited number of such promiscuous T-cell epitopes are known, and they will be discussed in detail below. It should be noted that in order for the immunogens which are used according to the present invention to be effective in as large a fraction of an animal population as possible, it may be 25 necessary to 1) insert several foreign T-cell epitopes in the same analogue or 2) prepare several analogues wherein each analogue has a different promiscuous epitope inserted. It should be noted that the concept of foreign T-cell epitopes also encompasses use of cryptic T-cell epitopes, i.e. epitopes 30 which are derived from a self-protein and which only exerts immunogenic behaviour when existing in isolated form without being part of the self-protein in question.

A "foreign T helper lymphocyte epitope" (a foreign T_H epitope) is a foreign T cell epitope which binds an MHC Class II molecule and can be presented on the surface of an antigen presenting cell (APC) bound to the MHC Class II molecule.

5 A "CTL" epitope is a peptide which is able to bind to an MHC class I molecule.

A "functional part" of a (bio)molecule is in the present context intended to mean the part of the molecule which is responsible for at least one of the biochemical or physiolo-
10 gical effects exerted by the molecule. It is well-known in the art that many enzymes and other effector molecules have an active site which is responsible for the effects exerted by the molecule in question. Other parts of the molecule may serve a stabilizing or solubility enhancing purpose and can
15 therefore be left out if these purposes are not of relevance in the context of a certain embodiment of the present invention. For instance it is possible to use certain cytokines as a modifying moiety in the analogue (cf. the detailed discussion below), and in such a case, the issue of stability may be
20 irrelevant since the coupling to the analogue provides the stability necessary.

The term "adjuvant" has its usual meaning in the art of vaccine technology, i.e. a substance or a composition of matter which is 1) not in itself capable of mounting a specific
25 immune response against the immunogen of the vaccine, but which is 2) nevertheless capable of enhancing the immune response against the immunogen. Or, in other words, vaccination with the adjuvant alone does not provide an immune re-
sponse against the immunogen, vaccination with the immunogen
30 may or may not give rise to an immune response against the immunogen, but the combined vaccination with immunogen and

adjuvant induces an immune response against the immunogen which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended to denote the situation where a molecule upon introduction in 5 the animal will appear preferentially in certain tissue(s) or will be preferentially associated with certain cells or cell types. The effect can be accomplished in a number of ways including formulation of the molecule in composition facilitating targeting or by introduction in the molecule of groups 10 which facilitates targeting. These issues will be discussed in detail below.

"Stimulation of the immune system" means that a substance or composition of matter exhibits a general, non-specific immunostimulatory effect. A number of adjuvants and putative 15 adjuvants (such as certain cytokines) share the ability to stimulate the immune system. The result of using an immunostimulating agent is an increased "alertness" of the immune system meaning that simultaneous or subsequent immunization with an immunogen induces a significantly more effective 20 immune response compared to isolated use of the immunogen

Preferred embodiments

In order to induce a CTL response against a cell which presents epitopes derived from the polypeptide antigen on its surface, it is normally necessary that at least one CTL epitope, when presented, is associated with an MHC Class I molecule on the surface of the APC. Furthermore it is preferred 25 that the at least one first foreign T_H epitope, when presented, is associated with an MHC Class II molecule on the surface of the APC.

Preferred APCs presenting the epitopes are dendritic cells and macrophages, but any pino- or phagocytizing APC which is capable of simultaneously presenting 1) CTL epitopes bound to MHC class I molecules and 2) T_H epitopes bound to MHC class II molecules, is a preferred APC according to the invention.

According to the invention, the cell-associated polypeptide antigen is preferably selected from a tumour-associated antigens and other self-proteins which are related to pathological processes but also viral antigens and antigens derived from an intracellular parasite or bacterium will. It is well-known in the art that such pathogen-associated antigens are often relatively poor immunogens (e.g. antigens from mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, but also from protozoans such as *Plasmodium spp.*). It is believed that the method of the invention, apart from rendering possible the production of antibody and CTL responses against true self-protein antigens, is capable of enhancing the often insufficient immune response mounted by the organism against such intracellular antigens.

Normally, it will be advantageous to confront the immune system with a large fraction of the amino acid sequence of the polypeptide antigen which is the vaccine target. Hence, in a preferred embodiment, presentation by the APC of the CTL epitope and the first foreign T_H epitope is effected by presenting the animal's immune system with at least one first analogue of the cell-associated polypeptide antigen, said first analogue comprising a variation of the amino acid sequence of the cell-associated polypeptide antigen, said variation containing at least the CTL epitope and the first foreign T_H epitope. This is in contrast to e.g. a DNA vaccination strategy where the CTL and T_H epitopes are expressed by the same cell but as parts of separate polypeptides; such a DNA vaccination strategy is also an embodiment of the invention,

but it is believed that having the two epitopes as part of the same polypeptide will normally enhance the immune response and, at any rate, the provision of only one expression product will be necessary.

- 5 In order to maximize the chances of mounting an effective immune response, it is preferred that the above-mentioned first analogue contains a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen, *i.e.* a fraction of the known and predicted CTL epitopes which binds a sufficient fractions of MHC Class I molecules in a population. For instance, it is preferred that the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 50% of the MHC-I haplotypes recognizing all known and predicted CTL epitopes in the cell-associated polypeptide antigen, but higher percentages are preferred, such as at least 60, at least 70, at least 80, and at least 90%. Especially preferred is the use of analogues which preserves substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue, *i.e.* close to 100% of the known CTL epitopes. Accordingly, it is also especially preferred that substantially all predicted CTL epitopes of the cell-associated polypeptide antigen are present in the at least first analogue.
- 25 Methods for predicting the presence of CTL epitopes are well-known in the art, cf. e.g. Rothbard *et al.* EMBO J. 7:93-100 (1988).

As will be apparent from the present specification and claims it is expected that the inventive method described herein will render possible the effective induction of CTL responses against cell-associated polypeptide antigens.

In cases where the cell-associated polypeptide antigen is truly intracellular, the induction of a CTL response against cells harbouring the antigen is the only way to achieve its down-regulation by specific immunological means. However, in 5 the case of membrane-associated antigens, it is advantageous to induce a antibody response against the weak, cell-associated polypeptide antigen. However, when raising a humoral immune response against a weak cell-associated antigen it is preferred to substantially restrict the antibody response to 10 interaction with the parts of the antigen which are normally exposed to possible interaction with antibodies. Otherwise the result would most likely be the induction of an antibody response against parts of the antigen which is not normally engaging the humoral immune system, and this will in turn 15 increase the risk of inducing cross-reactivity with antigens not related to any pathology. One elegant way of obtaining this restriction is to perform nucleic acid vaccination with an analogue of the weak cell-associated antigen, where the extracellular part thereof is either unaltered or includes a T_H 20 epitope which does not substantially alter the 3D structure of the extracellular part of the antigen. As one possible alternative, immunization can be performed with both a CTL directed immunogen and a B-cell directed immunogen where the B-cell directed immunogen is substantially incapable of effecting 25 immunization against the intracellular part of the target antigen (the B-cell directed immunogen could e.g. lack any non-extracellular material from the antigen).

Induction of antibody responses can be achieved in a number of ways known to the person skilled in the art. For instance, the 30 at least one first analogue may comprise a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-asso-

ciated polypeptide antigen - this variant is as mentioned above especially suited for nucleic acid vaccination. Alternatively, the method of the invention can involve effecting presentation to the animal's immune system of an immunogenically effective amount of at least one second analogue of the cell-associated polypeptide antigen which contains such a modification. A convenient way to achieve that the modification has the desired antibody-inducing effect is to include at least one second foreign T_H epitope in the second analogue,
5 i.e. a strategy like the one used for the first analogue.
10

In the cases where it is desired to also mount an effective humoral immune response, it is advantageous that the first and/or second analogue(s) comprise(s) a substantial fraction of the cell-associated polypeptide antigen's B-cell epitopes,
15 especially a substantial fraction of such B-cell epitopes which are extracellular in the naturally occurring form of the antigen in the pertinent animal.

The above-discussed variations and modifications of the weak, cell-associated polypeptide antigen can take different forms.
20 It is preferred that the variation and/or modification involves amino acid substitution and/or deletion and/or insertion and/or addition. These fundamental operations relating to the manipulation of an amino acid sequence are intended to cover both single-amino acid changes as well as operations
25 involving stretches of amino acids (i.a. shuffling of amino acid stretches within the polypeptide antigen; this is especially interesting when the antigen is a true intracellular antigen, since only considerations concerning preservation of CTL epitopes are relevant). It will be understood, that the
30 introduction of e.g. one single amino acid insertion or deletion may give rise to the emergence of a foreign T_H epitope in the sequence of the analogue, i.e. the emergence of an MHC Class II molecule binding sequence. However, in most situa-

tions it is preferable (and even necessary) to introduce a known foreign T_H epitope, and such an operation will require acid substitution and/or insertion (or sometimes addition in the form of either conjugation to a carrier protein or provision of a fusion polypeptide by means of molecular biology methods. It is preferred that the number of amino acid insertions, deletions, substitutions or additions is at least 2, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 25 insertions, substitutions, additions or 10 deletions. It is furthermore preferred that the number of amino acid substitutions is not in excess of 150, such as at most 100, at most 90, at most 80, and at most 70. It is especially preferred that the number of substitutions, insertions, deletions, or additions does not exceed 60, and in particular 15 the number should not exceed 50 or even 40. Most preferred is a number of not more than 30.

Preferred embodiments of the invention includes modification by introducing at least one foreign immunodominant T_H epitope. It will be understood that the question of immune dominance of 20 a T-cell epitope depends on the animal species in question. As used herein, the term "immunodominance" simply refers to epitopes which in the vaccinated individual/population gives rise to a significant immune response, but it is a well-known fact that a T-cell epitope which is immunodominant in one 25 individual is not necessarily immunodominant in another individual of the same species, even though it may be capable of binding MHC-II molecules in the latter individual. True immune dominant T_H epitopes are those which, independent of the polypeptide wherein they form a subsequence, give rise to 30 activation of T_H cells - in other words, some T_H epitopes have, as an intrinsic feature, the characteristic of substantially never being cryptic since they are substantially always processed by APCs and presented in the context of an MHC II molecule on the surface of the APC.

Another important point is the issue of MHC restriction of T-cell epitopes. In general, naturally occurring T-cell epitopes are MHC restricted, i.e. a certain peptides constituting a T-cell epitope will only bind effectively to a subset of MHC Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are variants of the antigen which are distinguished from each other by the nature of the T-cell epitope introduced.

If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

$$f_{population} = 1 - \prod_{i=1}^n (1 - p_i) \quad (II)$$

-where p_i is the frequency in the population of responders to the i^{th} foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-i.e. 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and DR7, then the use of this peptide together with another peptide which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the coverage at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula:

$$f_{population} = 1 - \prod_{j=1}^3 (1 - \varphi_j)^2 \quad (III)$$

-wherein φ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ); in practice, it is first determined which MHC molecules will recognize each T-cell epitope in the vaccine and thereafter these are listed by type (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding φ_1 , φ_2 , and φ_3 .

It may occur that the value p_i in formula II exceeds the corresponding theoretical value π_i :

$$\pi_i = 1 - \prod_{j=1}^3 (1 - \nu_j)^2 \quad (IV)$$

-wherein v_j is the sum of frequencies in the population of allelic haplotype encoding MHC molecules which bind the i^{th} T-cell epitope in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of 5 the population is a frequency of responders of $f_{\text{residual_}i} = (p_i - \pi_i) / (1-\pi_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$f_{\text{population}} = 1 - \prod_{j=1}^3 (1 - \varphi_j)^2 + \left(1 - \prod_{i=1}^n (1 - f_{\text{residual_}i}) \right) \quad (\text{V})$$

-where the term $1-f_{\text{residual_}i}$ is set to zero if negative. It 10 should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in 15 the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these 20 are preferably introduced in the vaccine thereby reducing the need for a very large number of different analogues in the same vaccine.

The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from 25 tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagglutinin (HA), and *P. falciparum* CS antigen.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible 5 T-cell epitopes to be introduced in analogues used according to the present invention. Cf. also the epitopes discussed in the following references which are hereby all incorporated by reference herein: WO 98/23635 (Frazer IH et al., assigned to The University of Queensland); Southwood S et. al, 1998, J. 10 Immunol. **160**: 3363-3373; Sinigaglia F et al., 1988, Nature **336**: 778-780; Rammensee HG et al., 1995, Immunogenetics **41**: 4 15 178-228; Chicz RM et al., 1993, J. Exp. Med **178**: 27-47; Hammer J et al., 1993, Cell **74**: 197-203; and Falk K et al., 1994, Immunogenetics **39**: 230-242. The latter reference also deals with HLA-DQ and -DP ligands. All epitopes listed in these 5 references are relevant as candidate natural epitopes to be used in the present invention, as are epitopes which share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epi- 20 tope which is capable of binding a large proportion of haplotypes. In this context the pan DR epitope peptides ("PADRE") described in WO 95/07707 and in the corresponding paper Alexander J et al., 1994, Immunity **1**: 751-761 (both disclosures are incorporated by reference herein) are interesting candi- 25 dates for epitopes to be used according to the present invention. It should be noted that the most effective PADRE peptides disclosed in these papers carry D-amino acids in the C- and N-termini in order to improve stability when administered. However, the present invention primarily aims at incorporating 30 the relevant epitopes as part of the modified antigen which should then subsequently be broken down enzymatically inside the lysosomal compartment of APCs to allow subsequent presentation in the context of an MHC-II molecule and therefore it

is not expedient to incorporate D-amino acids in the epitopes used in the present invention.

One especially preferred PADRE peptide is the one having the amino acid sequence AKFVAAWTLKAAA or an immunologically effective subsequence thereof. This, and other epitopes having the same lack of MHC restriction are preferred T-cell epitopes which should be present in the analogues used in the inventive method. Such super-promiscuous epitopes will allow for the most simple embodiments of the invention wherein only one single analogue is presented to the vaccinated animal's immune system.

The nature of the above-discussed variation/modification preferably comprises that

- at least one first moiety is included in the first and/or second analogue(s), said first moiety effecting targeting of the analogue to an antigen presenting cell (APC), and/or
- at least one second moiety is included in the first and/or second analogue(s), said second moiety stimulating the immune system, and/or
- at least one third moiety is included in the first and/or second analogue(s), said third moiety optimizing presentation of the analogue to the immune system.

The functional and structural features relating these first, second and third moieties will be discussed in the following:

They can be present in the form of side groups attached covalently or non-covalently to suitable chemical groups in the amino acid sequence of the cell-associated polypeptide antigen or a subsequence thereof. This is to mean that stretches of amino acid residues derived from the polypeptide antigen are derivatized without altering the primary amino acid sequence,

or at least without introducing changes in the peptide bonds between the individual amino acids in the chain.

The moieties can also be in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen. In this connection it should be mentioned that both possibilities include the option of conjugating the amino acid sequence to a carrier, cf. the discussion of these below. In other words, in the present context the term "fusion protein" is not merely restricted to a fusion construct prepared by means of expression of a DNA fragment encoding the construct but also to a conjugate between two proteins which are joined by means of a peptide bond in a subsequent chemical reaction.

As mentioned above, the analogue can also include the introduction of a first moiety which targets the analogue to an APC or a B-lymphocyte. For instance, the first moiety can be a specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific surface antigen. Many such specific surface antigens are known in the art. For instance, the moiety can be a carbohydrate for which there is a receptor on the B-lymphocyte or the APC (e.g. mannan or mannose). Alternatively, the second moiety can be a hapten. Also an antibody fragment which specifically recognizes a surface molecule on APCs or lymphocytes can be used as a first moiety (the surface molecule can e.g. be an FC γ receptor of macrophages and monocytes, such as FC γ RI or, alternatively any other specific surface marker such as CD40 or CTLA-4). It should be noted that all these exemplary targeting molecules can be used as part of an adjuvant, cf. below. CD40 ligand, antibodies against CD40, or variants thereof which bind CD40 will target the analogue to dendritic cells. At the same time, recent results have shown that the interaction with the CD40 molecule renders the T_H cells unessential for obtaining a CTL

response. Hence, it is contemplated that the general use of CD40 binding molecules as the first moiety (or as adjuvants, cf. below) will enhance the CTL response considerably; in fact, the use of such CD40 binding molecules as adjuvants and 5 "first moieties" in the meaning of the present invention is believed to be inventive in its own right.

As an alternative or supplement to targeting the analogue to a certain cell type in order to achieve an enhanced immune response, it is possible to increase the level of responsiveness 10 of the immune system by including the above-mentioned second moiety which stimulates the immune system. Typical examples of such second moieties are cytokines, heat-shock proteins, and hormones, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are 15 those which will normally also function as adjuvants in a vaccine composition, e.g. interferon γ (IFN- γ), Flt3 ligand (Flt3L), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and 20 granulocyte-macrophage colony stimulating factor (GM-CSF); alternatively, the functional part of the cytokine molecule may suffice as the second moiety. With respect to the use of such cytokines as adjuvant substances, cf. the discussion below.

25 Alternatively, the second moiety can be a toxin, such as listeriolycin (LLO), lipid A and heat-labile enterotoxin. Also, a number of mycobacterial derivatives such as MDP (muramyl dipeptide), CFA (complete Freund's adjuvant) and the trehalose diesters TDM and TDE are interesting possibilities.

According to the invention, suitable heat shock proteins used as the second moiety can be HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT).

Also the possibility of introducing a third moiety which
5 enhances the presentation of the analogue to the immune system
is an important embodiment of the invention. The art has shown
several examples of this principle. For instance, it is known
that the palmitoyl lipidation anchor in the *Borrelia burg-*
dorferi protein OspA can be utilised so as to provide self-
10 adjuvating polypeptides (cf. e.g. WO 96/40718). It seems that
the lipidated proteins form up micelle-like structures with a
core consisting of the lipidation anchor parts of the polypep-
tides and the remaining parts of the molecule protruding
therefrom, resulting in multiple presentations of the anti-
15 genic determinants. Hence, the use of this and related ap-
proaches using different lipidation anchors (e.g. a myristyl
group, a farnesyl group, a geranyl-geranyl group, a GPI-an-
chor, and an N-acyl diglyceride group) are preferred embodi-
ments of the invention, especially since the provision of such
20 a lipidation anchor in a recombinantly produced protein is
fairly straightforward and merely requires use of e.g. a
naturally occurring signal sequence as a fusion partner for
the analogue. Another possibility is use of the C3d fragment
of complement factor C3 or C3 itself (cf. Dempsey et al.,
25 1996, Science 271, 348-350 and Lou & Kohler, 1998, Nature
Biotechnology 16, 458-462).

It is important to note that when attempting to use the method
of the invention against e.g. membrane bound polypeptide
antigens which are exposed to the extracellular compartment,
30 it is most preferred that the first and/or second analogue(s)
has/have substantially the overall tertiary structure of the
cell-associated polypeptide antigen. In the present specifica-
tion and claims this is intended to mean that the overall

tertiary structure of the part of the polypeptide antigen which is extracellularly exposed is preserved, since, as mentioned above, the tertiary structure of the obligate intracellular polypeptides do not engage the humeral immune system. In fact, as part of the vaccination strategy it is often desired to avoid exposure to the extracellular compartment of putative B-cell epitopes derived from intracellular part of the polypeptide antigens; in this way, potentially adverse effects caused by cross-reactivity with other antigens can be minimized.

For the purposes of the present invention, it is however sufficient if the variation/modification (be it an insertion, addition, deletion or substitution) gives rise to a foreign T-cell epitope and at the same time preserves a substantial number of the CTL epitopes in the polypeptide antigen (and sometimes also a substantial number of B-cell epitopes).

The following formula describes the constructs generally covered by the invention:

$$(MOD_1)_{s1} (PAG_{e1})_{n1} (MOD_2)_{s2} (PAG_{e2})_{n2} \dots (MOD_x)_{sx} (PAG_{ex})_{nx} \quad (I)$$

-where PAG_{e1} - PAG_{ex} are x CTL and/or B-Cell epitope containing subsequences of the relevant polypeptide antigen which independently are identical or non-identical and which may contain or not contain foreign side groups, x is an integer ≥ 3 , $n1-nx$ are x integers ≥ 0 (at least one is ≥ 1), MOD_1 - MOD_x are x modifications introduced between the preserved epitopes, and $s1-sx$ are x integers ≥ 0 (at least one is ≥ 1 if no side groups are introduced in the sequences). Thus, given the general functional restraints on the immunogenicity of the constructs, the invention allows for all kinds of permutations of the original antigen sequence, and all kinds of modifications therein. Thus, included in the invention are analogues

obtained by omission of parts of the polypeptide antigen sequence which e.g. exhibit adverse effects in vivo or omission of parts which are normally intracellular and thus could give rise to undesired immunological reactions, cf. the detailed discussion below.

A further elaboration of the above principle include use of CTL and/or B-cell epitopes from more than one pathology-related antigen. For instance, there are several cancer related antigens that exert their oncogenic effects when they are in a mutated form only - examples are mutated K-ras and P53 which both are crucial proteins in normal cell cycle regulation and which both are expression products in most normal cells. In some cases, CTLs have been shown to recognise mutated peptides from these antigens. It is therefore important that the immune system responds to the mutated peptide only, and not to the unmutated parts, if antigen specific immunotherapy is instigated.

We have devised a strategy whereby sequences of 8-25 amino acids of such disease-related proteins could be used as further epitopes in an AutoVac construct - in preferred embodiments, the introduced epitopes would at the same time provide for the emergence of T_H epitopes in the final construct, cf. the discussion above. The epitopes used for this purpose would be those which comprise the mutated region of the disease-related protein. By using such an approach, it would be possible to generate CTLs (and possibly antibodies, where applicable) against only the mutated form of the disease-related antigen. In the cases where the disease-related antigen provides for the emergence of a T_H epitope, the use of a truly foreign T_H epitope could be completely omitted. An embodiment of this principle could e.g. be vaccination with a nucleic acid vaccine which encode an analogue of a polypeptide antigen (e.g. Her2 or PSM) wherein has been introduced at least one T_H

epitope and at least one peptide derived from another disease-related antigen (e.g. a peptide from the mutated part of an oncogenic protein). In a preferred embodiment, the at least one T_H epitope is introduced as a consequence of the introduction of the peptide.

It is furthermore preferred that the variation and/or modification includes duplication, when applicable, of the at least one B-cell epitope, or of at least one CTL epitope of the cell-associated polypeptide antigen. This strategy will give the result that multiple copies of preferred epitopic regions are presented to the immune system and thus maximizing the probability of an effective immune response. Hence, this embodiment of the invention utilises multiple presentations of epitopes derived from the polypeptide antigen (i.e. formula I wherein at least one B-cell epitope is present in two positions).

This effect can be achieved in various ways, e.g. by simply preparing fusion polypeptides comprising the structure (PAG)_m, where m is an integer ≥ 2 and then introduce the modifications discussed herein in at least one of the polypeptide antigen sequences.

An alternative embodiment of the invention which also results in the preferred presentation of multiple (e.g. at least 2) copies of the important epitopic regions of the antigen to the immune system is the covalent coupling of the antigen, subsequence or variants thereof to certain molecules. For instance, polymers can be used, e.g. carbohydrates such as dextran, cf. e.g. Lees A et al., 1994, Vaccine 12: 1160-1166; Lees A et al., 1990, J Immunol. 145: 3594-3600, but also mannose and mannan are useful alternatives. Integral membrane proteins from e.g. *E. coli* and other bacteria are also useful conjugation partners. The traditional carrier molecules such as

keyhole limpet haemocyanin (KLH), tetanus toxoid, diphtheria toxoid, and bovine serum albumin (BSA) are also preferred and useful conjugation partners.

Maintenance of the sometimes advantageous substantial fraction 5 of B-cell epitopes or even the overall tertiary structure of a protein which is subjected to modification as described herein can be achieved in several ways. One is simply to prepare a polyclonal antiserum directed against the polypeptide antigen (e.g. an antiserum prepared in a rabbit) and thereafter use 10 this antiserum as a test reagent (e.g. in a competitive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does the polypeptide antigen must be regarded as having the same overall tertiary structure as the polypeptide 15 antigen whereas analogues exhibiting a limited (but still significant and specific) reactivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive 20 with distinct epitopes on the polypeptide antigen can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of the polypeptide antigen in question and 2) a mapping of the epitopes which are maintained in the analogues prepared.

25 Of course, a third approach would be to resolve the 3-dimensional structure of the polypeptide antigen or of a biologically active truncate thereof (cf. above) and compare this to the resolved three-dimensional structure of the analogues prepared. Three-dimensional structure can be resolved by the 30 aid of X-ray diffraction studies and NMR-spectroscopy. Further information relating to the tertiary structure can to some extent be obtained from circular dichroism studies which have

the advantage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide and NMR requires the provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule.

5 However, ultimately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism can only provide indirect evidence of correct 3-dimensional structure via information of secondary structure elements.

10 In essence there are at present three feasible ways of obtaining the presentation of the relevant epitopes to the immune system: Traditional sub-unit vaccination with polypeptide antigens, administration of a genetically modified live vaccine, and nucleic acid vaccination. These three possibilities
15 will be discussed separately in the following:

Polypeptide vaccination

This entails administration to the animal in question of an immunogenically effective amount of the at least one first analogue, and, when relevant, administration of an immunologically effective amount of the at least one second analogue. Preferably, the at least one first and/or second analogue(s) is/are formulated together with a pharmaceutically and immunologically acceptable carrier and/or vehicle and, optionally an adjuvant.

25 When effecting presentation of the analogue to an animal's immune system by means of administration thereof to the animal, the formulation of the polypeptide follows the principles generally acknowledged in the art.

Preparation of vaccines which contain peptide sequences as
30 active ingredients is generally well understood in the art, as

exemplified by US Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms 5 suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients 10 are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the 15 vaccines; cf. the detailed discussion of adjuvants below.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously, intradermally, subdermally or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories 20 and, in some cases, oral, buccal, sublingual, intraperitoneal, intravaginal, anal and intracranial formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the 25 active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions 30 take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%. For oral formulations, cholera toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, 5 for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic 10 bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., 15 the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range 20 from about 0.1 µg to 2000 µg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 1000 µg, preferably in the range from 1 µg to 500 µg and especially in the range from about 10 µg to 100 µg. Suitable regimens for initial administration and booster shots 25 are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are 30 applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and

will vary according to the age of the person to be vaccinated and the formulation of the antigen.

Some of the polypeptides of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the 5 immune response will be enhanced if the vaccine further comprises an adjuvant substance. It is especially preferred to use an adjuvant which can be demonstrated to facilitate breaking of the autotolerance to autoantigens.

Various methods of achieving adjuvant effect for the vaccine 10 are known. General principles and methods are detailed in "The Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generation Immunological Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press, 15 New York, ISBN 0-306-45283-9, both of which are hereby incorporated by reference herein.

Preferred adjuvants facilitate uptake of the vaccine molecules by APCs, such as dendritic cells, and activate these. Non-limiting examples are selected from the group consisting of an 20 immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ -inulin; and 25 an encapsulating adjuvant. In general it should be noted that the disclosures above which relate to compounds and agents useful as first, second and third moieties in the analogues also refer *mutatis mutandis* to their use in the adjuvant of a vaccine of the invention.

30 The application of adjuvants include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05

to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C
5 for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-
10 negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Admixture with oils such as squalene and IFA is also preferred.

15 According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant as is DNA and γ -inulin, but also Freund's complete and incomplete adjuvants as well as quillaja saponins such as QuilA and QS21 are interesting. Further possibilities are monophosphoryl lipid A
20 (MPL), and the above mentioned C3 and C3d.

Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are preferred according to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix)
25 adjuvants are preferred choices according to the invention, especially since it has been shown that this type of adjuvants are capable of up-regulating MHC Class II expression by APCs. An ISCOM® matrix consists of (optionally fractionated) saponins (triterpenoids) from Quillaja saponaria, cholesterol, and
30 phospholipid. When admixed with the immunogenic protein, the resulting particulate formulation is what is known as an ISCOM particle where the saponin constitutes 60-70% w/w, the choles-

terol and phospholipid 10-15% w/w, and the protein 10-15% w/w. Details relating to composition and use of immunostimulating complexes can e.g. be found in the above-mentioned text-books dealing with adjuvants, but also Morein B et al., 1995, Clin.

5 Immunother. 3: 461-475 as well as Barr IG and Mitchell GF, 1996, Immunol. and Cell Biol. 74: 8-25 (both incorporated by reference herein) provide useful instructions for the preparation of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility
10 of achieving adjuvant effect is to employ the technique described in Gosselin et al., 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant antigen such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen
15 binding antibody fragments) against the Fc γ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-Fc γ RI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of the targeting and
20 immune modulating substances (i.a. cytokines) mentioned above as candidates for the first and second moieties in the modified analogues. In this connection, also synthetic inducers of cytokines like poly I:C are possibilities.

Suitable mycobacterial derivatives are selected from the group
25 consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the group consisting of CD40 ligand and CD40 antibodies or specifically binding fragments thereof (cf. the discussion above),
30 mannose, a Fab fragment, and CTLA-4.

Suitable polymer adjuvants are selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a plastic polymer; and latex such as latex beads.

5 Yet another interesting way of modulating an immune response is to include the immunogen (optionally together with adjuvants and pharmaceutically acceptable carriers and vehicles) in a "virtual lymph node" (VLN) (a proprietary medical device developed by ImmunoTherapy, Inc., 360 Lexington Avenue, New York, NY 10017-6501). The VLN (a thin tubular device) mimics the structure and function of a lymph node. Insertion of a VLN under the skin creates a site of sterile inflammation with an upsurge of cytokines and chemokines. T- and B-cells as well as APCs rapidly respond to the danger signals, home to the inflamed site and accumulate inside the porous matrix of the VLN. It has been shown that the necessary antigen dose required to mount an immune response to an antigen is reduced when using the VLN and that immune protection conferred by vaccination using a VLN surpassed conventional immunization using Ribi as an adjuvant. The technology is *i.a.* described briefly in Gelber C et al., 1998, "Elicitation of Robust Cellular and Humoral Immune Responses to Small Amounts of Immunogens Using a Novel Medical Device Designated the Virtual Lymph Node", in: "From the Laboratory to the Clinic, Book of Abstracts, October 12th - 15th 1998, Seaside Resort, Aptos, California".

Recent findings have demonstrated that the co-administration of H2 agonists enhances the in-tumour survival of Natural Killer Cells and CTLs. Hence, it is also contemplated to 30 include H2 agonists as adjuvants in the methods of the invention.

It is expected that the vaccine should be administered at least once a year, such as at least 1, 2, 3, 4, 5, 6, and 12 times a year. More specifically, 1-12 times per year is expected, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times 5 a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred autovaccines according to the invention is not permanent, and therefor the immune system needs to be periodically challenged with the analogues.

10 Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response, cf. also the discussion above concerning the choice 15 of foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above.

The vaccine may consequently comprise 3-20 different modified or unmodified polypeptides, such as 3-10 different polypeptides. 20 However, normally the number of peptides will be sought kept to a minimum such as 1 or 2 peptides.

Live vaccines

The second alternative for effecting presentation to the immune system is the use of live vaccine technology. In live 25 vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic microorganism which has been transformed with a nucleic acid fragment encoding the necessary epitopic regions or a complete 1st and/or 2nd analogue. Alternatively, the microorganism is transformed with 30 a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacte-

rial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*,
5 *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. 45: 1492-1496 and Walker PD, 1992, Vaccine 10: 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live
10 vaccines, cf. the discussion below.

As for the polypeptide vaccine, the T_H epitope and/or the first and/or second and/or third moieties can, if present, be in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen.

15 As an alternative to bacterial live vaccines, the nucleic acid fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector. One possibility is a pox virus such as vaccinia, MVA (modified Vaccinia virus), canary pox, avi-pox, and chicken pox etc. Alternatively, a
20 herpes simplex virus variant can be used.

Normally, the non-pathogenic microorganism or virus is administered only once to the animal, but in certain cases it may be necessary to administer the microorganism more than once in a lifetime.

25 Also, the microorganism can be transformed with nucleic acid(s) containing regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses
30 having the coding region for the analogue and the coding region for the immunomodulator in different open reading

frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitopes are produced as fusion partners to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used as 5 transforming agents.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation", 10 "gene immunisation" and "DNA vaccination) offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the 15 form of industrial scale fermentation of microorganisms producing the analogues necessary in polypeptide vaccination). Furthermore, there is no need to devise purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of 20 the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant 25 fraction of the original B-cell epitopes should be preserved in the analogues derived from extracellularly exposed polypeptide sequences, and since B-cell epitopes in principle can be constituted by parts of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and 30 lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is best ensured by having the host producing the immunogen.

Hence, an important embodiment of the method of the invention involves that presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment which encodes and expresses the at least one CTL epitope and/or the at least 5 one B-cell epitope, and the at least one first foreign T_H epitope (an alternative encompasses administration of at least 2 distinct nucleic acid fragments, where one encodes the at least one CTL epitope and the other encodes the at least one foreign T_H epitope). Preferably, this is done by using a nu- 10 cleic acid fragment which encodes and expresses the above- discussed first analogue. If the first analogue is equipped with the above-detailed T_H epitopes and/or first and/or second and/or third moieties, these are then present in the form of fusion partners to the amino acid sequence derived from the 15 cell-associated polypeptide antigen, the fusion construct being encoded by the nucleic acid fragment.

As for the traditional vaccination approach, the nucleic acid vaccination can be combined with *in vivo* introduction, into the APC, of at least one nucleic acid fragment encoding and 20 expressing the second analogue. The considerations pertaining to 1st, 2nd and 3rd moieties and T_H epitopes apply also here.

In this embodiment, the introduced nucleic acid is preferably DNA which can be in the form of naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA 25 included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, and DNA formulated with an adjuvant. In this 30 context it is noted that practically all considerations pertaining to the use of adjuvants in traditional vaccine formulation apply for the formulation of DNA vaccines. Hence, all disclosures herein which relate to use of adjuvants in the

context of polypeptide based vaccines apply *mutatis mutandis* to their use in nucleic acid vaccination technology. The same holds true for other considerations relating to formulation and mode and route of administration and, hence, also these 5 considerations discussed above in connection with a traditional vaccine apply *mutatis mutandis* to their use in nucleic acid vaccination technology.

One especially preferred type of formulation of nucleic acid vaccines are microparticles containing the DNA. Suitable 10 microparticles are e.g. described in WO 98/31398.

Furthermore, the nucleic acid(s) used as an immunization agent can contain regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A 15 preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different open reading frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitope is produced as a fusion partner to the immunomodulator. Alternatively, two distinct 20 nucleotide fragments can be used, but this is less preferred because of the advantage of ensured co-expression when having both coding regions included in the same molecule.

Under normal circumstances, the nucleic acid of the vaccine is 25 introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al., 30 1997, Annu. Rev. Immunol. 15: 617-648 and Donnelly JJ et al., 1997, Life Sciences 60: 163-172. Both of these references are incorporated by reference herein.

An important part of the invention pertains to a novel method for selecting an appropriate immunogenic analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said immunogenic analogue being 5 capable of inducing a CTL response in the animal against cells displaying an MHC Class I molecule bound to an epitope derived from the cell-associated polypeptide antigen. This method comprises the steps of

- a) identifying at least one subsequence of the amino acid sequence of the cell-associated polypeptide antigen, 10 where said subsequence does not contain known or predicted CTL epitopes,
- b) preparing at least one putatively immunogenic analogue of the cell-associated polypeptide antigen by introducing, 15 in the amino acid sequence of the cell-associated polypeptide antigen, at least one T_H epitope foreign to the animal in a position within the at least one subsequence identified in step a), and
- c) selecting the/those analogues prepared in step b) which 20 are verifiably capable of inducing a CTL response in the animal.

Alternatively, the above selection method involves the preparation of a nucleic acid fragment for nucleic acid vaccination purposes. In that situation, it is required that the encoded 25 peptide includes at least one T_H epitope.

When the analogue is derived from an antigen which is exposed to the extracellular phase, it is preferred that the subsequence identified in step a) further does not contain cysteine residues, or, alternatively, that the T_H epitope introduced in 30 step b) does not substantially alter the pattern of cysteine residues. This approach facilitates the preservation of spatial B-cell epitopes in the resulting construct which are

similar to the B-cell epitopes in the weak, cell-associated polypeptide antigen.

For the same reasons it is preferred that the subsequence identified in step a) further does not contain known or predicted glycosylation sites, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the glycosylation pattern.

Certain of the weak, cell-associated polypeptide antigens exert undesired effects by having a pathophysiological role.

10 It is desired that these effects are not exerted by the vaccination constructs, and therefore it is preferred that the subsequence identified in step a) contributes significantly to a pathophysiological effect exerted by the cell-associated polypeptide antigen, and that the introduction in step b) of 15 the foreign T_H epitope reduces or abolishes said pathophysiological effect. An example of this approach is to remove the active site in an enzyme, hormone or cytokine and exchange this with the foreign T_H epitope.

Another important consideration pertains to the question of 20 immunological cross-reactivity of the vaccine's polypeptide product with other self-proteins which are not related to a pathology. Such cross-reactivity should preferably be avoided and hence an important embodiment of this method of the invention is one where the subsequence identified in step a) is 25 homologous to an amino acid sequence of a different protein antigen of the animal, and where the introduction of the T_H epitope in step b) substantially removes the homology.

Related to this embodiment is an embodiment where any amino acid sequences which 1) are not normally exposed to the 30 extracellular phase and 2) which may constitute B-cell epitopes of the weak, cell-associated polypeptide antigen, are

not preserved in the analogue. This can be achieved by exchanging such amino acid sequences with T_H epitopes which do not constitute B-cell epitopes, by completely removing them, or by partly removing them.

- 5 On the other hand, it is preferred that any "true" B-cell epitopes of the weak cell-associated polypeptide antigen are preserved to a high degree, and therefore an important embodiment of the selection method of the invention involves that the introduction in step b) of the foreign T_H epitope results
- 10 in preservation of a substantial fraction of B-cell epitopes of the cell-associated polypeptide antigen. It is especially preferred that the analogue preserves the overall tertiary structure of the cell-associated polypeptide antigen.

The preparation in step b) is preferably accomplished by molecular biological means or by means of solid or liquid phase peptide synthesis. Shorter peptides are preferably prepared by means of the well-known techniques of solid- or liquid-phase peptide synthesis. However, recent advances in this technology has rendered possible the production of full-length polypeptides and proteins by these means, and therefore it is also within the scope of the present invention to prepare the long constructs by synthetic means.

After having identified the useful analogues according to the above-discussed method, it is necessary to produce the analogue in larger scale. The polypeptides are prepared according to methods well-known in the art.

This can be done by molecular biological means comprising a first step of preparing a transformed cell by introducing, into a vector, a nucleic acid sequence encoding an analogue which has been selected according to the method and transforming a suitable host cell with the vector. The next step is to

culture the transformed cell under conditions facilitating the expression of the nucleic acid fragment encoding the analogue of the cell-associated antigen, and subsequently recovering the analogue from the culture supernatant or directly from the 5 cells, e.g. in the form of a lysate). Alternatively, the analogue can be prepared by large-scale solid or liquid phase peptide synthesis, cf. above.

Finally, the product can, depending on the cell chosen as a host cell or the synthesis method used, be subjected to artificial post-translational modifications. These can be refolding schemes known in the art, treatment with enzymes (in order to obtain glycosylation or removal of undesired fusion partners, chemical modifications (again glycosylation is a possibility), and conjugation, e.g. to traditionally carrier 15 molecules.

It should be noted that preferred analogues of the invention (and also the relevant analogues used in the methods of the invention) comprise modifications which results in a polypeptide having a sequence identity of at least 70% with the 20 polypeptide antigen or with a subsequence thereof of at least 10 amino acids in length. Higher sequence identities are preferred, e.g. at least 75% or even at least 80% or 85%. The sequence identity for proteins and nucleic acids can be calculated as $(N_{ref} - N_{dif}) \cdot 100 / N_{ref}$, wherein N_{dif} is the total number of 25 non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$).

Specific exemplary targets for the method of the invention

As discussed above, preferred weak, cell-associated polypeptide antigens are tumour-associated antigens. A non-limiting list of these are given in the following table.

<u>Antigen</u>	<u>Reference</u>
5 alpha reductase	Délos S, Carsol JL, Fina F, Raynaud JP, Martin PM. 5alpha-reductase and 17beta-hydroxysteroid dehydrogenase expression in epithelial cells from hyperplastic and malignant human prostate. Int J Cancer 1998 Mar 16 75:6 840-6
α -fetoprotein	Esteban C, Terrier P, Frayssinet C, Uriel J. Expression of the alpha-fetoprotein gene in human breast cancer. Tumour Biol 1996 17:5 299-305
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CD19	Scheuermann RH, Racila E. CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy. Leuk Lymphoma 1995 Aug 18:5-6 385-97
CD20	Knox SJ, Goris ML, Trisler K, Negrin R, Davis T, Liles TM, Grillo-López A, Chinn P, Varns C, Ning SC, Fowler S, Deb N, Becker M, Marquez C, Levy R. Yttrium-90-labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma. Clin Cancer Res 1996 Mar 2:3 457-70
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CD46	Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M, Imai Y. Protection of thyroid cancer cells by complement-regulatory factors. Cancer 1994 Jun 1 73:11 2808-17
CD5	Stein R, Witz IP, Ovadia J, Goldenberg DM, Yron I. CD5+ B cells and naturally occurring autoantibodies in cancer patients. Clin Exp Immunol 1991 Sep 85:3 418-23
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CD55 (791Tgp72)	Spendlove, I., L. Li, J. Carmichael, & L. G. Durrant. Decay accelerating factor (CD55): A target for cancer vaccine? (1999) Cancer Research 59: 2282-2286.
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DcR3	Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Pitti R et al, Nature 396, 699-703. 1998.
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EGFR	Yang XD, Jia XC, Corvalan JR, Wang P, Davis CG, Jakobovits A: Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. Cancer Res 1999, 59(6):1236-43.
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Folic Acid Receptor	Dixon KH, Mulligan T, Chung KN, Elwood PC, Cowan KH. Effects of folate receptor expression following stable transfection into wild type and methotrexate transport-

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G250	deficient ZR-75-1 human breast cancer cells. <i>J Biol Chem</i> 1992 Nov 25 267:33 24140-7 Divgi CR, Bander NH, Scott AM, O'Donoghue JA, Sgouros G, Welt S, Finn RD, Morrissey F, Capitelli P, Williams JM, Deland D, Nakhre A, Oosterwijk E, Gulec S, Graham MC, Larson SM, Old LJ. Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. <i>Clin Cancer Res</i> 1998 Nov 4:11 2729-39
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gastrin 17	Watson SA, Michaeli D, Grimes S, Morris TM, Crosbee D, Wilkinson M, Robinson G, Robertson JF, Steele RJ, Hardcastle JD. Anti-gastrin antibodies raised by gastrimmune inhibit growth of the human colorectal tumour AP5. <i>Int J Cancer</i> 1995 Apr 10 61:2 233-40 Wang QJ, Knezetic JA, Schally AV, Pour PM, Adrian TE. Bombesin may stimulate proliferation of human pancreatic cancer cells through an autocrine pathway. <i>Int J Cancer</i> 1996 Nov 15 68:4 528-34
GD2 / GD3 / GM2	Wiesner DA, Sweeley CC. Circulating gangliosides of breast-cancer patients. <i>Int J Cancer</i> 1995 Jan 27 60:3 294-9
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GP1	
gp100 / Pmel 17	Wagner SN, Wagner C, Schultewolter T, Goos M. Analysis of Pmel17/gp100 expression in primary human tissue specimens: implications for melanoma immuno- and gene-therapy. <i>Cancer Immunol Immunother</i> 1997 Jun 44:4 239-47 Kirkin AF, Dzhandzhugazy K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. <i>APMIS</i> 1998 Jul 106:7 665-79
gp-100-in4	

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gp15	Maeurer MJ, et al. New treatment options for patients with melanoma: review of melanoma-derived T-cell epitope-based peptide vaccines. <i>Melanoma Res.</i> 1996 Feb;6(1):11-24.
gp75 / TRP-1	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. <i>Semin Cancer Biol</i> 1995 Dec 6:6 321-7
hCG	Hoermann R, Gerbes AL, Spoettl G, Jüngst D, Mann K. Immunoreactive human chorionic gonadotropin and its free beta subunit in serum and ascites of patients with malignant tumors. <i>Cancer Res</i> 1992 Mar 15 52:6 1520-4
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Her2 / neu	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. <i>Semin Cancer Biol</i> 1995 Dec 6:6 321-7
HMTV	Kahl LP, Carroll AR, Rhodes P, Wood J, Read NG. An evaluation of the putative human mammary tumour retrovirus associated with peripheral blood monocytes. <i>Br J Cancer</i> 1991 Apr 63:4 534-40
Hsp70	Jaattela M, et al. Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. <i>EMBO J.</i> 1998 Nov 2;17(21):6124-34.
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IGFR1	M.J. Ellis et. al., Insulin-like growth factors in human breast cancer. <i>Breast Cancer Res. Treat.</i> (1998) 52; 175-184.
IL-13R	Murata T, Obiri NI, Debinski W, Puri RK. Structure of IL-13 receptor: analysis of subunit composition in cancer and immune cells. <i>Biochem Biophys Res Commun</i> 1997 Sep 8 238:1 90-4
iNOS	Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. <i>Cancer</i> 1998 May 15 82:10 1897-903
Ki 67	Gerdes, J., U. Schwab, H. Lemke, and H. Stein. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell prolifera-

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K-ras, H-ras, N-ras	Abrams SI, Hand PH, Tsang KY, Schlom J. Mutant ras epitopes as targets for cancer vaccines. Semin Oncol 1996 Feb 23:1 118-34
KSA (CO17-1A)	Zhang S, Zhang HS, Reuter VE, Slovin SF, Scher HI, Livingston PO. Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. Clin Cancer Res 1998 Feb 4:2 295-302
LDLR-FUT	Caruso MG, Osella AR, Notarnicola M, Berloco P, Leo S, Bonfiglio C, Di Leo A. Prognostic value of low density lipoprotein receptor expression in colorectal carcinoma. Oncol Rep 1998 Jul-Aug 5:4 927-30
MAGE Family (MAGE1, MAGE3)	Marchand M, et al., Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999 Jan 18;80(2):219-30.
Mammaglobin	Watson MA, Dintzis S, Darrow CM, Voss LE, DiPersio J, Jensen R, Fleming TP, Mammaglobin expression in primary, metastatic, and occult breast cancer. Cancer Res 1999 Jul 1 59:13 3028-31.
MAP17	Kocher O, Cheresh P, Lee SW. Identification and partial characterization of a novel membrane-associated protein (MAP17) up-regulated in human carcinomas and modulating cell replication and tumor growth. Am J Pathol 1996 Aug 149:2 493-500
Melan-A / MART-1	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. Semin Cancer Biol 1995 Dec 6:6 321-7
mesothelin	Chang K, et al. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc Natl Acad Sci U S A. 1996 Jan 9;93(1):136-40
MIC A/B	Groh V, Steinle A, Bauer S, and Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science 1998, 279: 1737-1740.
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Mox1	Candia AF, Hu J, Crosby J, Lalley PA, Noden D, Nadeau JH, Wright CV. Mox-1 and Mox-2 define a novel homeobox

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p15	Yoshida S, Todoroki T, Ichikawa Y, Hanai S, Suzuki H, Hori M, Fukao K, Miwa M, Uchida K. Mutations of p16Ink4/CDKN2 and p15Ink4B/MTS2 genes in biliary tract cancers. Cancer Res 1995 Jul 1 55:13 2756-60
P170 / MDR1	Trock BJ, Leonessa F, Clarke R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst 1997 Jul 2 89:13 917-31
p53	Roth J, Dittmer D, Rea D, Tartaglia J, Paoletti E, Levine AJ. p53 as a target for cancer vaccines: recombinant canarypox virus vectors expressing p53 protect mice against lethal tumor cell challenge. Proc Natl Acad Sci U S A 1996 May 14 93:10 4781-6.
p97 / melanotransferrin	Furukawa KS, Furukawa K, Real FX, Old LJ, Lloyd KO. A unique antigenic epitope of human melanoma is carried on the common melanoma glycoprotein gp95/p97. J Exp Med 1989 Feb 1 169:2 585-90
PAI-1	Grøndahl-Hansen J, Christensen IJ, Rosenquist C, Brünner N, Mouridsen HT, Danø K, Blichert-Toft M. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993 Jun 1 53:11 2513-21
PDGF	Vassbotn FS, Andersson M, Westermark B, Heldin CH, Ostman A. Reversion of autocrine transformation by a dominant negative platelet-derived growth factor mu-

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Plasminogen (uPA)	tant. Mol Cell Biol 1993 Jul 13:7 4066-76 Naitoh H, Eguchi Y, Ueyama H, Kodama M, Hattori T. Localization of urokinase-type plasminogen activator, plasminogen activator inhibitor-1, 2 and plasminogen in colon cancer. Jpn J Cancer Res 1995 Jan 86:1 48-56
PRAME	Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998 Jul 106:7 665-79
Probasin	Matuo Y, Nishi N, Muguruma Y, Yoshitake Y, Kurata N, Wada F. Localization of prostatic basic protein (''probasin'') in the rat prostates by use of monoclonal antibody. Biochem Biophys Res Commun 1985 Jul 16 130:1 293-300
Progenipoitin PSA	Sanda MG, Smith DC, Charles LG, Hwang C, Pienta KJ, Schliom J, Milenic D, Panicali D, Montie JE. Recombinant vaccinia-PSA (PROSTVAC) can induce a prostate-specific immune response in androgen-modulated human prostate cancer. Urology 1999 Feb 53:2 260-6.
PSM	Kawakami M, Nakayama J. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by <i>in situ</i> hybridization. Cancer Res 1997 Jun 15 57:12 2321-4
RAGE-1	Gaugler B, 7 others, Van den Eynde BJ. A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma. Immunogenetics 1996;44(5):323-330.
Rb	Dosaka-Akita H, Hu SX, Fujino M, Harada M, Kinoshita I, Xu HJ, Kuzumaki N, Kawakami Y, Benedict WF. Altered retinoblastoma protein expression in nonsmall cell lung cancer: its synergistic effects with altered ras and p53 protein status on prognosis. Cancer 1997 Apr 1 79:7 1329-37
RCAS1	Sonoda K, Nakashima M, Kaku T, Kamura T, Nakano H, Watanabe T. A novel tumor-associated antigen expressed in human uterine and ovarian carcinomas. Cancer 1996 Apr 15;77(8):1501-1509.
SART-1	Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H, Itoh K. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer 1999 May 5 81:3 459-66
SSX gene family	Gure AO, Tüüreci O, Sahin U, Tsang S, Scanlan MJ, Jäger E, Knuth A, Pfreundschuh M, Old LJ, Chen YT. SSX: a

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STn (mucin assoc.)	Sandmaier BM, Oparin DV, Holmberg LA, Reddish MA, MacLean GD, Longenecker BM. Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with Theratope STn-KLH cancer vaccine. J Immunother 1999 Jan 22:1 54-66
TAG-72	Kuroki M, Fernsten PD, Wunderlich D, Colcher D, Simpson JF, Poole DJ, Schlom J. Serological mapping of the TAG-72 tumor-associated antigen using 19 distinct monoclonal antibodies. Cancer Res 1990 Aug 15 50:16 4872-9
TGF- α	Imanishi K, Yamaguchi K, Suzuki M, Honda S, Yanaihara N, Abe K. Production of transforming growth factor-alpha in human tumour cell lines. Br J Cancer 1989 May 59:5 761-5
TGF- β	Picon A, Gold LI, Wang J, Cohen A, Friedman E. A subset of metastatic human colon cancers expresses elevated levels of transforming growth factor beta1. Cancer Epidemiol Biomarkers Prev 1998 Jun 7:6 497-504
Thymosin β 15	Bao, L., Loda, M., Janmey, P. A., Stewart, R., Anand-Apte, B., and Zetter, B. R. Thymosin beta 15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nature Medicine. 2 (12), 1322-1328. 1996
TNF- α	Moradi MM, Carson LF, Weinberg B, Haney AF, Twiggs LB, Ramakrishnan S. Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer. Cancer 1993 Oct 15 72:8 2433-40
TPA	Maulard C, Toubert ME, Chretien Y, Delanian S, Dufour B, Housset M. Serum tissue polypeptide antigen (S-TPA) in bladder cancer as a tumor marker. A prospective study. Cancer 1994 Jan 15 73:2 394-8
TPI	Nishida Y, Sumi H, Mihara H. A thiol protease inhibitor released from cultured human malignant melanoma cells. Cancer Res 1984 Aug 44:8 3324-9
TRP-2	Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma

Antigen	Reference
Tyrosinase	antigen tyrosinase-related protein 2 (TRP2). Cancer Res 1998 Nov 1 58:21 4895-901 Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998 Jul 106:7 665-79
VEGF	Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, Jinno K, Kotani Y. Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer 1998 Dec 34:13 2041-5
ZAG	Sanchez LM, Chirino AJ, Bjorkman Pj. 1999, Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. Science 19;283(5409):1914-9
p16INK4	Quelle DE, Ashmun RA, Hannon GJ, Rehberger PA, Trono D, Richter KH, Walker C, Beach D, Sherr CJ, Serrano M. Cloning and characterization of murine p16INK4a and p15INK4b genes. Oncogene 1995 Aug 17;11(4):635-45
Glutathione S-transferase	Hengstler JG, Arand M, Herrero ME, Oesch F. Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. Recent Results Cancer Res 1998 154: 47-85

In the following, a number of specific tumour-associated antigens will be discussed in detail.

Prostate-specific membrane antigen, PSM

In U.S.A., prostate cancer is the second leading cause of 5 cancer death (app. 40,000 per year), and 200,000 patients per year are diagnosed (Boring 1993). Approximately 1 out of 11 men eventually will develop prostatic cancer. Furthermore, approximately 40-60% of prostate cancer patients eventually develop extraprostatic extension of the disease (Babaian 10 1994). The main strategy in the present invention is to use a therapeutic vaccine as a supplementary therapy to prostatectomy in order to eliminate residual tumour tissue and metastases.

Several pathologic conditions are located to the prostate gland, including benign growth (BPH), infection (prostatitis) and neoplasia (prostatic cancer).

The biological aggressiveness of prostatic cancer is variable.

- 5 In some patients the detected tumour remains a latent histologic tumour and never becomes clinically significant. In other patients, the tumour progresses rapidly, metastasises and kills the patient in a relatively short time period (2-5 years).
- 10 The current primary treatment of prostate cancer is prostatectomy. However, due to the extensive spreading of prostate cancer cells the majority of prostatic cancer patients are not cured by local surgery. Patients with non-confined disease eventually receive systemic androgen ablation therapy, but the
- 15 annual death rate from prostatic cancer has not declined at all over the 50 years since androgen ablation became standard therapy for metastatic disease.

PSM is a membrane protein which is highly specific for prostatic tissues, benign as well as malignant, although expression of PSM has also been observed in other tissues such as renal tissue and renal tumor, small intestine, brain and tumor neovasculature. Therefore, if surgery was successful, prostatectomised cancer patients should theoretically express PSM on residual malignant prostate tumour tissue or metastases originating from the tumour. By inducing a strong CTL response and/or a strong polyclonal antibody response towards PSM, it is expected that residual tumour tissue can be eliminated.

Interestingly, upregulation of PSM expression is seen following androgen-deprivation therapy of prostate cancer patients (Wright 1996). This would make a PSM-targeted treatment very

well-suited to follow the traditional androgen-deprivation therapy.

PSM was first identified in 1987 as a result of generating a monoclonal antibody, 7E11-C5.3, raised against an isolated 5 human prostatic cancer cell, LNCaP (Horoszewicz 1987). The antibody recognised both normal and malignant prostatic epithelium, and was used in 1993 to purify and determine the amino acid sequence of the PSM protein and eventually clone the gene (Israeli 1993).

10 PSM is a type II transmembrane glycoprotein with a molecular weight of 84 kD as predicted from the nucleic acid sequence whereas the glycosylated version has an observed molecular weight of 100-120 kD. Sequencing of the gene encoding PSM revealed a putative membrane spanning region in connection
15 with three cytosolic arginine anchor residues. The extracellular part of PSM constitute 707 of the total 750 amino acids of the protein, whereas the cytoplasmic domain is predicted to be 19 amino acids long (Israeli 1993). PSM-specific mRNA has been detected in prostate tumour tissue (Israeli 1994), indicating
20 that the tumour antigen is not an aberrantly glycosylated protein which is the case with e.g. the Tn- or sTn-tumour antigens.

The full length PSM cDNA has been transfected into and expressed in a PSM negative human prostate cancer cell line, PC-25 3 (Kahn 1994). Furthermore, the full length (2.65 kilobases) cDNA has been transcribed and translated *in vitro* (Kahn 1994).

It has recently been demonstrated that PSM possesses hydrolytic activity resembling that of the N-acetylated α -linked acidic dipeptidase (NAALADase) - in fact it has been demonstrated that the two proteins are identical. NAALADase is a membrane-bound hydrolase of the nervous system, which

catabolises the neuropeptide N-acetylaspartyl glutamate (NAAG) in order to affect the glutamatergic signalling processes. It is still not known whether this activity of PSM has any relevant biological function.

- 5 It is of some importance to predict whether undesired cross-reactivity with other proteins accessible for CTLs or antibodies would be expected following treatment with an autovaccine inducing PSM-specific immune responses. It has been shown that a part of the coding region of the PSM gene (amino acids
- 10 positions 418-567) has 54% homology to the human transferrin receptor (Israeli 1993). Also, complete sequence identity with the NAALADase enzyme has been found, cf. above. No identification of a functionally relevant similarity with other known peptidases could be made.
- 15 The homology to the transferrin receptor is very low and will preferably be disrupted in some of the inventive constructs. The observed sequence identity with human NAALADase is not expected to be an obstacle for a PSM-vaccine, partly because of the low ability of antibodies and CTLs to penetrate the
- 20 blood-brain barrier. Altogether, even with the most PSM-like construct, it is not expected to experience prohibitive cross-reactivity with other proteins in the patients.

From earlier studies it is clear that PSM is expressed on most prostate cancer cells and prostate originating metastases
25 tested. Further, most other cancers tested, such as carcinomas, sarcomas and melanomas of different tissues as well as a large panel of non-prostatic human cancer cell lines have proven PSM negative.

In addition to this, a very large number of other tissues have
30 been found to be PSM negative. These include colon, breast, lung, ovary, liver, urinary bladder, uterus, bronchus, spleen,

pancreas, tongue, esophagus, stomach, thyroid, parathyroid, adrenal, lymph node, aorta, vena cava, skin, mammary gland and placenta. However, RT-PCR has revealed the existence of PSM mRNA in some of these tissues.

5 Although PSM is predominantly found as a membrane bound molecule on prostate tissue small amounts of PSM can also be detected in the sera of normal individuals and in elevated levels in prostate cancer patients (Rochon 1994, Murphy 1995). The level of circulating PSM in these patients therefore
10 allows a serological monitoring of the effectiveness of a PSM vaccine.

In conclusion, based on the entire amount of data available to date, PSM is an antigen with a high specificity for human prostate tissue and tumours originating therefrom. This means
15 that in patients who have undergone prostatectomy, PSM is a tumour quasi-specific self-antigen. An effective PSM vaccine is therefore likely to target mainly prostatic or prostate-originating metastatic tissue.

As will be clear from Example 1 the method of the invention
20 preferably entails that foreign T_H-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699. Furthermore, a modified PSM
25 molecule which has a foreign T_H-epitope introduced in these positions is also a part of the invention.

Accordingly, the invention also pertains to an analogue of human PSM which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and
30 B-cell epitopes of PSM and including at least one foreign T_H epitope as discussed herein. Preferred PSM analogues are those

wherein the at least one foreign T_H epitope is present as an insertion in the PSM amino acid sequence or as a substitution of part of the PSM amino acid sequence or as the result of deletion of part of the PSM amino acid sequence, and most preferred analogues are those wherein a foreign T_H-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699.

10 *Human Chorionic Gonadotropin (HCG)*

The relationship between embryonic markers and malignant phenotypes has been under discussion for many decades. An increasing body of data suggests that at least one such marker, human chorionic gonadotropin beta (hCG β), is consistently detected on cancer cells of many different histological origins, and that expression of this protein often correlates with increased metastatic properties. A humoral immune response directed against this soluble protein may reduce the chances of tumour spreading and/or may inhibit the recurrence 20 of new primary growths post-surgery.

Human chorionic gonadotropin belongs to a family of glycoprotein hormones, including follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH), all of which are important regulators of reproductive 25 expression and fetal survival. The members of this family of hormones are heterodimers, which share a common α -chain. The β -chain is unique to each hormone and provides the specificity, with the β -chain of LH exhibiting the strongest sequence homology to hCG β (approximately 80%). The apparent molecular 30 weight of hCG-holo, is 37 kD, of which one third is contributed by carbohydrate. The post-translational sugar modifications include both N-linked and O-linked carbohydrate. Abun-

dant sialic acid residues are present and these give the protein a large negative charge. The crystal structure of hCG-holo has been solved (Lapthorn *et al.*, 1994).

Based on the crystal structure it was found that hCG exhibits 5 homology to a family of growth factors, including PDGF and TGF β (Lapthorn *et al.*, 1994). This suggests that hCG expression may help regulating cancer cell growth.

Human chorionic gonadotropin is a glycoprotein hormone, which is produced by the placental syncytiotrophoblasts soon after 10 conception, and it is essential for successful gestation in the pregnant woman.

The pathophysiological role of embryonic markers for the development or maintenance of a cancer mass is not known. However, it is of interest to note that trophoblasts (where 15 these proteins are normally produced) have both *angiogenic* and *invasive* characteristics, both of which are also necessary properties for a cancer cell. Further, it has been suggested that hCG (or its subunits) can inhibit maternal cellular immune responses to fetal tissue. For example, studies have 20 shown that hCG directly suppresses T cell responses (Jacoby *et al.*, 1984) and it has been proposed that because the lymph nodes draining (in this case) a primary melanoma tumour, are immunosuppressed, a more favourable environment for metastatic tumours to establish themselves may result. As a consequence, 25 expression of hCG β may help cancer cells spread into the secondary lymphoid organs. Finally, as mentioned above, structural homology between hCG and a number of growth factors have been demonstrated. Another possibility is therefore that secretion of hCG by cancer cells may give the tumour a growth 30 advantage.

Expression of hCG β has been shown in many different types of cancer, for example: a) Prostate adenocarcinoma: positivity for hCG β on tissue sections were seen for patients with poor prognosis, irrespective of the histological grade of the
5 tumour (Sheaff et al., 1996), b) different kinds of lung carcinomas; squamous cell (SQCC), adenocarcinoma (AC), and large cell (LCC), all showed a high percentage of reactivity for hCG β (Boucher et al. 1995), c) pancreatic adenocarcinoma (Syrigos et al., 1998), d) neuroblastomas, brain cancers,
10 retinoblastomas (Acevedo et al., 1997), e) malignant melanoma (Doi et al., 1996), f) bladder carcinomas (Lazar et al., 1995). A recent paper describes a DNA approach, in which mice were immunized with a hCG β expression construct (Geissler et al., 1997). In this *in vivo* model inhibition of tumour growth
15 was strongly associated with CTL-activity, however high titers of antibodies (which neutralized the biological effect of intact hCG on its cellular receptor) were also detected.

The use of hCG as an immunogen has been described in several papers, focussing on its use as a contraceptive vaccine (Talwar et al., 1976 and Talwar et al., 1994). A very high degree of efficacy and safety has been observed in an anti-fertility clinical trial, using a vaccine against hCG-holo (Talwar et al., 1994). Phase I clinical trials of cancer patients with a vaccine against a synthetic carboxy-terminal peptide of hCG β
25 conjugated to diphtheria toxoid have also been conducted (Triozzi et al., 1994) and phase II trials are underway. Despite the fact that the idea to use hCG β as a cancer vaccine target has been around for some time, it has not been explored in conjunction with the AutoVac technology.

30 It is known that cells from non-embryonic tissue, or benign neoplasms, do not express hCG β . Therefore, there should be no potential side effects from vaccination against this molecule (apart from the effects on pregnancy). Because it is expressed

by so many different kinds of cancers this molecule has been proposed to be the "definitive cancer biomarker" (Acevedo *et al.*, 1995 and Regelson W., 1995) and as such would be an attractive target to go after.

5 Suitable animal models for Further studies of the efficacy of a hCG based vaccine can be found in Acevedo *et al.*, Cancer Det. and Prev. Suppl. (1987) 1: 477-486, and in Kellen *et al.*, Cancer Immunol. Immunother. (1982) 13: 2-4.

Her2

10 The tyrosine kinase receptors Her2 and EGFr are believed to play a crucial role in the malignant transformation of normal cells and in the continued growth of cancer cells. Overexpression is usually linked to a very poor prognosis. During the past few years there has been many reports concerning the use
15 of antibodies against these receptors as therapy for cancers that overexpress either or both of these receptors. Genentech Inc. has finished several successful clinical trials on breast cancer patients using a monoclonal antibody against Her2 and has recently obtained an FDA approval for the marketing of the
20 anti-Her2 monoclonal antibody preparation, Herceptin®.

The autovaccination technology disclosed herein as applied on the Her2 molecule would elicit polyclonal antibodies that would predominantly react with Her2. Such antibodies are expected to attack and eliminate tumour cells as well as
25 prevent metastatic cells from developing into metastases. The effector mechanism of this anti-tumour effect would be mediated via complement and antibody dependent cellular cytotoxicity.

Dependent on the choice of constructs, the induced autoantibodies could also inhibit cancer cell growth through inhibition
30

of growth factor dependent oligo-dimerisation and internalisation of the receptors. And, most importantly, the Her2 analogues are expected to be able to induce CTL responses directed against known and/or predicted Her2 epitopes displayed
5 by the tumour cells

Her2 is a member of the epidermal growth factor receptor family (c-erbB) which consists of four different receptors to date: c-erbB-1 (EGFr), c-erbB-2 (Her2, c-Neu), c-erbB-3 and c-erbB-4 (Salomon et al, 1995). C-erbB-3 and c-erbB-4 are less well characterised than EGFr and Her2. Her2 is an integral glycoprotein. The mature protein has a molecular weight of 185 kD with structural features that closely resembles the EGFr receptor (Prigent et al, 1992). EGFr is also an integral membrane receptor consisting of one subunit. It has an apparent molecular weight of 170 kD and consists of a surface ligand-binding domain of 621 amino acids, a single hydrophobic transmembrane domain of 23 amino acids, and a highly conserved cytoplasmic tyrosine kinase domain of 542 amino acids. The protein is N-glycosylated (Prigent et al, 1994).

All proteins in this family are tyrosine kinases. Interaction with the ligand leads to receptor dimerisation, which increases the catalytic action of the tyrosine kinase (Bernard. 1995, Chantry 1995). The proteins within the family are able to homo- and heterodimerise which is important for their activity. The EGFr conveys growth promoting effects and stimulates uptake of glucose and amino acids by cells (Prigent et al 1992). Her2 also conveys growth promoting signals. Only EGFr binds EGF and TGF-alpha. These ligands do not bind to the other receptors in the family (Prigent et al., 1992). The ligands for Her2 are not fully determined. However, heregulin has been shown to induce phosphorylation by activating Her2. This does not appear to be due to a direct binding to the receptor but it is believed that heregulin is a ligand for

erbB-3 and erbB-4 which then activates Her2 by oligo-dimerisation (*Solomon et al 1995*).

The homology between the proteins of EGF receptor family is most pronounced in the tyrosine kinase domain at the cytoplasmic part of the molecules (82% between EGFr and Her2). The homology is less in the extracellular part - from 41% to 46% in different domains (*Prigent et al, 1992*).

The epidermal growth factor receptor is expressed on normal tissues in low amounts, but it is overexpressed in many types of cancers. EGFr is overexpressed in breast cancers (*Earp et al, 1993, Eppenberger 1994*), gliomas (*Schlegel et al, 1994*), gastric cancer (*Tkunaga et al, 1995*), cutaneous squamous carcinoma (*Fujii 1995*), ovarian cancer (*van Dam et al, 1994*) and others. Her2 is also expressed on few normal human tissues in low amount, most characteristically on secretory epithelia. Over expression of Her2 occurs in about 30% of breast, gastric, pancreatic, bladder and ovarian cancers.

The expression of these receptors varies depending on the degree of differentiation of the tumours and the cancer type, e.g., in breast cancer, primary tumours overexpress both receptors; whereas in gastric cancer, the overexpression occurs at a later stage in metastatic tumours (*Salomon et al, 1995*). The number of overexpressed receptors on carcinoma cells is greater than 10^6 /cell for several head and neck cancers, vulva, breast and ovarian cancer lines isolated from patients (*Dean et al, 1994*).

There are several reasons why the EGFr family of receptors constitute suitable targets for tumour immunotherapy. First, they are overexpressed in many types of cancers, which should direct the immune response towards the tumour. Second, the tumours often express or overexpress the ligands for this

family of receptors and some are hypersensitive to the proliferative effects mediated by the ligands. Third, patients with tumours that overexpress growth factor receptors often have a poor prognosis. The overexpression has been closely linked with poor prognosis especially in breast cancer, lung cancer and bladder cancer (2) and is apparently associated with invasive/metastatic phenotypes, which are rather insensitive to conventional therapies (*Eccles et al, 1994*).

Overexpression of Her2 is in some cases a result of amplification of the gene and in other cases increased transcription and translation. The overexpression of Her2 is associated with poor prognosis in breast, ovarian cancers, gastric cancer, bladder cancer and possibly in non-small cell lung cancers (*Solomon et al, 1995*).

Phase I clinical trials have been performed with a bispecific antibody in patients with advanced breast and ovarian cancer. The antibody was bispecific against Her2 and Fc_YRI (*Weiner et al, 1995*). Efficient lysis of Her2 over expressing tumour cells was observed with a bispecific antibody against Her2 and CD3 (*Zhu et al, 1995*).

Treatment of scid mice xenografted with human gastric cancer with an anti-Her2 monoclonal antibody prolonged the survival of the mice (*Ohniski et al, 1995*). The anti-tumour activities of monoclonal antibodies against Her2, *in vitro* and *in vivo* is not due to an identical mechanism; they may act as partial ligand agonists, alter Her2 receptor turnover and phosphorylation or may affect dimerization (*Lupu et al, 1995*).

Similarly, it has been shown that antibodies to EGFr can also interfere with growth factor interactions. (*Baselga et al, 1994, Modjahedi et al, 1993a, Wu et al, 1995, Modjahedi et al,*

1993b, Tosi et al, 1995, Dean et al, 1994, Bier et al, 1995, Modjtahedi et al, 1996, Valone 1995).

Hence, an important embodiment of the methods of the invention is one wherein the foreign T-cell epitope is introduced in a 5 part of the Her2 amino acid sequence defined by the amino acid numbering in SEQ ID NO: 3 positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-593 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or 10 710-730, cf. the Examples.

Accordingly, the invention also relates to an analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign 15 T_H epitope as discussed herein. It is preferred that the at least one foreign T_H epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of the Her2 amino acid sequence. Most preferred analogues are 20 those defined above, i.e. those wherein the foreign T-cell epitope is introduced in a part of the Her2 amino acid sequence defined by SEQ ID NO: 3 positions positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-25 593 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or 710-730.

FGF8b

It has been shown by several investigators that FGF8b can induce proliferation, transformation, differentiation and in 30 some cases greatly increase the tumorigenicity of mammalian cells and tissues (Tanaka 1992, Kouhara 1994, Lorenzi 1995,

MacArthur 1995a, Crossley 1996a, 1996b, Ghosh 1996, Ohuchi 1997a, Rudra-Ganguly 1998). These effects are primarily mediated through the binding of FGF8b to members of the fibroblast growth factor receptors FGFR2, FGFR3, and FGFR4 (MacArthur 1995b, Blunt 1997, Tanaka 1998). Thus, cells expressing one of these receptors and FGF8(b) have been shown to provide an autocrine growth-signaling cascade leading to proliferation. The biological effect of FGF8b is most likely partly mediated through the JAK/STAT3 pathway, since we and others have observed that addition of FGF8b to the growth medium of certain cells does promote phosphorylation of STAT3, a feature suspected to render cells resistant to apoptosis (Catlett-Falcone 1999).

In addition to the in vitro observations mentioned above, it has recently been shown that FGF8(b) expression is significantly upregulated in both prostate and breast cancers (Marsh 1999, Dorkin 1999). We therefore believe, that an autovaccine against FGF8(b) will be a very efficient means of treating a number of FGF8-expressing tumors, or perhaps increase their sensitivity towards apoptosis inducing agents.

Prostate cancer

The biological aggressiveness of prostatic cancer is variable. In some patients the detected tumor remains a latent histologic tumor and never becomes clinically significant. In other patients, the tumor progresses rapidly, metastasizes, and kills the patient in a relatively short time (2-5 years).

For the purpose of diagnosis, and to follow the response to therapy of prostate cancer, determination of the circulating levels of two proteins has primarily been used: prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (Nguyen 1990, Henttu 1989). Due to disruption of the normal architec-

ture of the prostate gland in response to cancer development, these soluble proteins are released into the circulation where they can be detected as markers for e.g. metastatic spread.

The current primary treatment of prostate cancer is prostatectomy. However, due to the extensive spreading of prostate cancer cells the majority of prostatic cancer patients are not cured by local surgery. Patients with non-confined disease receive systemic androgen ablation therapy, but the annual death rate from prostatic cancer has not declined at all over 10 the 50 years since androgen ablation became standard therapy for metastatic disease.

RT-PCR analysis has shown that FGF8 mRNA is produced by the human prostatic epithelial tumor cell lines LNCaP, PC-3, ALVA-31, and DU145 respectively, with FGF8b being the most prominent isoform (Tanaka 1995, Ghosh 1996). The growth of the androgen-responsive LNCaP cells are stimulated by addition of recombinant FGF8b (Tanaka 1995), while DU145 cells could be growth inhibited by transfection with virus expressing anti-sense FGF8b (Rudra-Ganguly 1998). This, together with 20 evidence from developmental studies discussed below, indicate a role for FGF8b in maintaining the cancerous state of these cell lines.

Using FGF8a cDNA for in situ hybridization experiments, Leung and co-workers have shown that a high proportion (80% (n=106), 25 and 71% (n=31)) of prostatic cancers produce FGF8 mRNA, and that the amount of FGF8 mRNA correlate with the severeness of the tumors ($P<0.0016$, and $P<0.05$, respectively) (Leung 1996, Dorkin 1999). Using a monoclonal anti-FGF8b antibody, this isoform was shown responsible for the overexpression of FGF8b 30 (Dorkin 1999). Additionally, men with tumors which expressed high levels of FGF8 had worse survival ($P=0.034$), and that FGF8 expression persisted in androgen independent prostate

cancers (Dorkin 1999). According to the data presented by Dorkin and coworkers the expression of FGF8b in prostate cancer could predict patient survival.

Immunohistochemical analysis using a monoclonal antibody 5 against FGF8, has detected the protein in 93% (n=43) of human prostate cancers (Tanaka 1998). Normal prostatic tissue or benign prostatic hyperplasia does produce low levels of FGF8 mRNA, and does not contain detectable amounts of FGF8 protein (Leung 1996, Yoshimura 1996, Ghosh 1996, Tanaka 1998, Dorkin 10 1999).

These results indicate that an autovaccine against FGF8(b) would be reactive against prostatic tumor tissue and thus, extremely valuable in the treatment of prostatic cancer.

Breast cancer

15 The current treatment of breast cancer is surgery. However, due to the extensive spreading of breast cancer cells a large part of breast cancer patients are not cured by local surgery. Patients with non-confined disease eventually receive androgen ablation therapy, chemotherapy, and or radiation therapy. The 20 annual death rate from breast cancer is, however, still relatively high.

FGF8 was originally isolated from a mouse mammary carcinoma cell-line (SC-3), from which the expression could be induced by adding androgen to the medium (Nonomura 1990). The protein 25 is also known to induce the proliferation of these as well as other mammalian cells. Recently FGF8b mRNA has been shown to be present in eight (n=8) human breast cancer cell lines (MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42, HBL-100 and MCF-7) (Tanaka 1995, Payson 1996, Wu 1997, Marsh 30 1998).

Wnt-1 transgenic mice infected with mouse mammary tumor virus (MMTV) develop mammary tumors. FGF8 transcription is activated in 50% of these tumors (MacArthur 1995c, Kapoun 1997).

Transgenic mice that are carrying the FGF8b cDNA under control
5 of the very specific mouse mammary tumor virus (MMTV) pro-
moter, are shown to spontaneously develop FGF8b expressing
mammary tumors (Coombes, personal communication).

Very recent data shows that FGF8(b) expression is upregulated
in breast cancer (Tanaka 1998, Marsh 1999). Tanaka and co-
10 workers used a new monoclonal FGF8 antibody in
immunohistochemical studies. They showed that FGF8 was present
in 67% (n=12) of breast cancers, and that androgen receptors
were present in 89% of FGF8 positive breast diseases (Hyper-
plasia, Fibroadenoma, Intraductal papilloma, and cancers),
15 which would allow the autocrine growth promoting loop to be
involved in the progression of breast cancers (Tanaka 1998).
Using a semi-quantitative RT-PCR method, it was shown that
elevated levels of FGF8 mRNA were found in malignant compared
to non-malignant breast tissues. Significantly more malignant
20 tissues were expressing FGF8 ($p=0.019$) at significantly higher
levels ($p=0.031$) (68 breast cancers and 24 non-malignant
breast tissues) (Marsh 1999).

It has not yet been fully established that FGF8(b) functions
as an autocrine growth factor. However, the fact that a large
25 number of tumors overexpress FGF8b argues strongly that an
autovaccine against FGF8b could be effective against a large
percentage of breast and prostate cancers. The data reported
by Marsh, Dorkin, and Tanaka indicate that an autovaccine
against FGF8(b) could be used for treatment of both breast and
30 prostate cancers, and the rather vague data presented by
Dorkin et. al, is a further support of the opinion that FGF8
is involved in the proliferation of human cancer cells.

Description of FGF8b

FGF8 belongs to the family of fibroblast growth factors (FGFs). These growth regulatory proteins are small ~200 amino acid residue proteins that all are involved in the induction 5 of proliferation and differentiation of a wide range of cells. For a recent review of the involvement of the fibroblast growth factors in vertebrate limb development, see Johnson 1997. The FGF family members are evolutionary related and share 20-50% amino acid sequence identity.

10 FGF8b is a splice variant of FGF8, originally termed androgen induced growth factor (AIGF). AIGF was first identified as a protein secreted by a murine mammary carcinoma derived cell line (SC-3) upon stimulation with androgen (Nonomura 1990). The murine FGF8 gene contains 6 exons, potentially coding for 15 eight different FGF8-isoforms (FGF8a-h), differing only in the N-terminal part of the molecules (Crossley 1995, MacArthur 1995b). Human FGF8 has the same gene structure as the murine gene. However, due to a stop codon in exon 1B, human FGF8 can possibly exist in four different isoforms namely FGF8a, FGF8b, 20 FGF8e, and FGF8f (Gemel 1996). The gene structures and the amino acid sequences of the four human isoforms are illustrated in Fig. 5.

Mature FGF8b contains 193 amino acid residues, and has a calculated molecular weight of 22.5kDa. The highly basic 25 protein contains 21 arginine and 14 lysine residues resulting in a calculated isoelectric point of 10.84, and a calculated positive charge of 19,8 at pH 7.0. It contains two cysteine residues, and has two potential N-glycosylation sites. Due to the nature of the investigations performed involving FGF8b 30 very little is known about the FGF8b protein moiety. It has, however, been expressed heterologously from bacteria, purified by the use of a C-terminal hexa-Histidine tag, and *in vitro*

refolded to a soluble and biologically active state (MacArthur 1995a, Blunt 1997).

Biological activity of FGF8b

As mentioned above, FGF8(b) was first isolated as a factor
5 that was released from a androgen dependent mouse mammary
tumor cell line, and it has been shown that this protein can
induce the proliferation of these cells. The morphological
changes mimic those induced by testosterone, which is also
know to induce the synthesis of FGF8(b) mRNA (Tanaka 1992).

10 The proliferation can be inhibited by FGF8(b) antisense oligos
(Nonomura 1990, Tanaka 1992, and Yamanishi 1994). Indeed, a
human prostate cancer cell line DU145 could be growth inhi-
bited by transfection with vira expressing anti-sense FGF8b
(Rudra-Ganguly 1998). Recent data shows that FGF8b induces
15 phosphorylation of STAT3 - a protein that is suspected to be
involved in resistance to apoptosis (Catlett-Falcone 1999,
Johnston, C.L., unpublished results).

FGF8b has by several investigators been shown very efficient
in inducing the transformation of NIH3T3 or SC115 cells (Miya-
20 shita 1994, Kouhara 1994, Lorenzi 1995, MacArthur 1995a). By
using recombinantly expressed proteins, it has also been shown
that this induction of morphological changes is far more
efficient with FGF8b than when using FGF8a or FGF8c (MacArthur
1995a, Ghosh 1996). Interestingly, the N-terminal half of the
25 FGF8b molecule alone, was shown to be sufficient for transfor-
mation of NIH3T3 cells, and even the small FGF8b specific
peptide (QVTVQSSPNFT) could enable the cells to grow 2-3 times
longer than normal in 0.1% serum (Rudra-Ganguly 1998). Fur-
thermore, NIH3T3 cells stably transfected with an expression
30 vector encoding FGF8b has been reported to be very tumorigenic
when injected intraocularly into nude mice (Kouhara 1994,
Ghosh 1996).

In vivo, FGF8b is known to be expressed at certain stages of development in vertebrates. A summary of the biological roles assigned to FGF8(b) is shown in Table 1. For reviews on the involvement of FGF8 in vertebrate development see Goldfarb 5 1999, and Johnson 1997.

Table: Various sites/tissues known to produce FGF8, and the proposed biological role(s).

	Action/mechanism/presence (species)	References
	Present in the developing limb buds (mouse)	Heikinheimo
		1994, Ohuchi
10	Limb bud outgrowth (chicken)	Kuwana 1997, Xu 1998
	Induction of ectopic limb formation from mesoderm (chicken)	Crossley 1996b
	Induction of midbrain formation from the caudal diencephalon (chicken)	Crossley 1996a
15	Initiation of wing outgrowth in a wingless mutant (chicken)	Ohuchi 1997a
	Role in dorsoventral patterning of the gastrula (zebrafish)	Fürthauer 1997
	Required during gastrulation, cardiac,	Meyers 1998
20	craniofacial, forebrain, midbrain and cerebellar development (tissue specific knock-out mice)	
	Role in tooth morphogenesis (mouse)	Kettunen 1998

It is believed that FGF8(b) mediates its action through binding to the fibroblast growth factor receptors (FGFR's). Specifically, FGF8b is known to be able to activate FGFR2c, FGFR3c, FGFR4c, and to some extent also FGFR1c, but not FGFR1b, -2b or -3b (MacArthur 1995b, Blunt 1997). In case of the induction of outgrowth of ectopic chicken limbs, it is 25 implicated that FGF10, FGFR2, and FGF8 interact and that this could be sufficient for outgrowth (Kuwana 1997, Xu 1998). 30

These results support the hypothesis that FGF8(b) can act in an auto- and paracrine manner, leading to the normal outgrowth and patterning of several anatomical structures during vertebrate development. Importantly, FGF8 "total knock out" mice do 5 not survive most likely due to the elaborate involvement of the protein in the development of the embryo.

Homology to other proteins

It is of significant importance to predict whether undesired cross-reactivity with other proteins accessible for antibodies 10 would be expected following treatment with an autovaccine inducing FGF8b specific autoantibodies. Due to the high degree of sequence identity between FGF8b and the other FGF8 molecules, an autovaccine will be expected to cross-react with these proteins. This, however, will presumably be advantageous, since none of these proteins are reported to be expressed in tissues or by cell-lines that do not already express FGF8b.

Amino acid residues 55 through 175 of FGF8b shows a relatively low but significant degree of sequence identity to the other 20 FGFs. It is commonly accepted (and several times proven) that a significant degree of sequence identity between two protein domains is also reflected in a high degree of tertiary structure similarity. Therefore, the FGF family members are all generally expected to be structurally similar. The three 25 dimensional structure of FGF2 has been resolved from crystals as well as in solution (Ago 1991, Zhang 1991, Zhu 1991, Eriksson 1993, Blaber 1996, Moy 1996). FGF2 is composed entirely of beta-sheet structure, comprising a three-fold repeat of a four-stranded antiparallel beta-meander. This beta-barrel 30 structure is totally conserved between interleukin 1, FGF2 (or basic FGF), and FGF1 (or acidic FGF). Nuclear magnetic resonance analysis of FGF2 in solution has shown that the N-termi-

nal part of the molecule forms a relatively flexible structure. The remaining part of FGF8b (amino acid residues 1-54 and 176-215) only shows a low degree of sequence identity to known proteins.

5 Based on the structural and alignment data, it is generally assumed that the three dimensional structural core of the other fibroblast growth factors closely resemble those of FGF1 and FGF2. These structural considerations are important factors in our design of the FGF8b mutant autovaccine molecules.

10

Importantly, due to the relatively low degree of sequence identity between FGF8 and any of the other members of the FGF family, the surface of FGF8 would be very different from that of other FGFs, thereby minimizing the cross-reactivity of

15 FGF8b autovaccine generated antibodies with other FGF family members. Due to the very low degree of homology to other proteins than the fibroblast growth factors, we do not expect an autovaccine against FGF8b to cross-react with any other proteins.

20 It should be emphasized, however, that an autovaccine against FGF8b probably would cross react with all isoforms of FGF8. This will, however, presumably not be a problem since none of the FGF8 isoforms are expected to be expressed at significant levels in the adult. It is even possible that this cross reaction will be beneficial in the treatment of cancer, since 25 it has been shown that at least some cancer cell lines express other isoforms in addition to FGF8b.

Tissue distribution of FGF8b

Ideally, the induced autoantibodies and the subsequent effector mechanisms as well as the expected CTL response raised by 30 autovaccination should only be directed towards tissues that

are to be eliminated in the patient. Therefore, the tissue distribution of the antigen, which is targeted by an autovaccine, is an issue of great importance concerning the safety of the vaccine.

5 Table: Expression of FGF8b in various tissues and cells

Human

	Breast cancer cell lines (MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42, HBL-100, and MCF-7)	((RT-PCR) Tanaka 1995, Pay- son 1996, Wu 1997, Marsh 1999)
10	Breast tumors	((mAb) Tanaka 1998, (RT-PCR) Marsh 1999)
	Normal breast tissue	((RT-PCR) Wu 1997, Marsh 1999 (mAb) Tanaka 1998)
	Prostate cancer (93%)	((in situ hyb.) Leung 1996, Dorkin 1999, (mAb) Tanaka 1998)
	Breast disease	((mAb) Tanaka 1998)
15	Prostatic tumor cells (LNCaP, PC-3, DU145, and ALVA-31) fetal kidney	((RT-PCR) Tanaka 1995, Ghosh 1996, Schmitt 1996) ((Northern blot) Ghosh 1996)
	adult prostate, testis, kid- ney, neurons	((RT-PCR) Ghosh 1996, Wu 1997, Dorkin 1999)
20	teratocarcinoma cells (Tera-2)	((RT-PCR) Wu 1997)

Murine

	Breast cancer cell lines (SC-115, RENCA)	((RT-PCR) Yoshimura 1996)
	Hypothalamus, Testis	((RT-PCR) Yoshimura 1996)
25	Mammary tumors (Wnt-1 trans- genic)	((Northern blot) MacArthur 1995c)
	Embryonic brain	((in situ hyb.) Crossley 1995, Heikinheimo 1994, Ohuchi 1994, Shimamura 1997, (RT-PCR) Blunt 1997)
	Ovary, testis	((Northern blot) Valve 1997)
	Developing face and limb buds	((pAb) MacArthur 1995b (in situ hyb.) Heikinheimo 1994, Ohuchi 1994, Crossley 1995)
30	Gastrula	((in situ hyb.) Crossley 1995)

Chicken

	Embryonic brain	((in situ hyb.) Crossley 1996a)
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developing limb buds ((*in situ* hyb.) Ohuchi
1997a,b)

Rat

Prostate and testis (RT-PCR) Scmitt 1996

The above table shows a wide selection of tissue distribution,
5 and cell line data of FGF8b expression. As seen from the
table, most of the data regarding tissue distribution is
generated using the sensitive RT-PCR method. This is because
Northern blotting analysis does not detect any FGF8b mRNA in
any normal tissues except from fetal kidney. From this scarce
10 data, it is generally assumed that expression of FGF8b mRNA in
the adult is very limited, and thus, an autovaccine against
FGF8b would presumably not be reactive against normal tissue.
Due to the fact that small amounts of FGF8b could interact in
unknown systems in the adult, the tissue distribution of the
15 protein needs further analysis. There are, however, in our
opinion no indications that an autovaccine against FGF8b would
result in serious unwanted effects on the patients.

Effects of antibodies against FGF8b

So far, no attempts to treat prostate cancer using monoclonal
20 antibodies have been published. Clinical trials with monoclo-
nal antibodies are ongoing in breast cancer therapy studies,
however.

Antibodies against FGF8b will probably block the interaction
between FGF8b and its receptors, which will inhibit the cell
25 membrane ruffling and cell proliferation, very likely
decreasing the motility and invasiveness of the cancer cells.

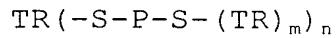
Hence, the invention also relates to embodiments of the
methods described herein where, where the foreign T-cell
epitope is introduced in a part of the FGF8b amino acid se-

quence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177. It
5 should be noted that it is especially preferred *not* to introduce variations or modifications in positions 26-45 and in the C-terminus starting at amino acids 186-215, since these stretches show the least homology with a recently discovered protein, FGF-18, which seems to be expressed in a variety of
10 non-tumour tissues.

Accordingly, the invention also pertains to an analogue of human/murine FGF8b which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of FGF8b and including at least one
15 foreign T_H epitope as discussed herein. It is preferred that the at least one foreign T_H epitope is present as an insertion in the FGF8b amino acid sequence or as a substitution of part of the FGF8b amino acid sequence or as the result of deletion of part of the FGF8b amino acid sequence. Most preferred
20 analogues in this embodiment are those where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or
25 138-144 and/or 149-154 and/or 158-162 and/or 173-177.

Mucins

The invention also pertains to methods of the invention employing specifically modified versions of the human mucins, especially any of MUC-1 through MUC-4, preferably MUC-1. The
30 analogues comprise the following structure



-where TR is a tandem repeat derived from the naturally occurring mucin, P is a foreign T_H-epitope as discussed herein, S is an inert spacer peptide having from 0 to 15 amino acid residues, preferably between 0 and 10 amino acid residues, and n
5 is an integer of from 1 to 30, and m is an integer from 1 to 10, preferably from 3 to 5.

When producing such a mucin analogue in e.g. a human cell line or by purification from a tissue, the direct result will normally not have a glycosylation pattern as desired, i.e. an
10 aberrant glycosylation pattern resembling that of a tumour derived mucin. However, it is possible to produce the analogue recombinantly in e.g. *E. coli* or by synthetic means, and subsequently glycosylating the product enzymatically so as to achieve a Tn or S-Tn glycosylation pattern specific for MUC-1
15 expressed on tumours. Alternatively, the polypeptide could be prepared in a mammalian cell line or an insect cell line, e.g. *Drosophila* cells, which lacks the relevant enzyme or by expressing the protein intracellularly (by omitting a secretion signal peptide) where glycosylation does not occur.

20 Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that the analogues can be prepared by means of recombinant gene technology but also by means of chemical synthesis or semisynthesis; the latter two options are especially relevant when the
25 modification consists in coupling to protein carriers (such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and non-proteinaceous molecules such as carbohydrate polymers and of course also when the modification comprises addition of side chains or side groups to an polypeptide-derived peptide chain.

30 For the purpose of recombinant gene technology, and of course also for the purpose of nucleic acid immunization, nucleic

acid fragments encoding the necessary epitopic regions and analogues are important chemical products. Hence, an important part of the invention pertains to a nucleic acid fragment which encodes an analogue described above of any of the relevant 5 tumour-specific polypeptides, preferably a polypeptide wherein has been introduced a foreign T_H-cell epitope by means of insertion and/or addition, preferably by means of substitution and/or deletion. The nucleic acid fragments of the invention are either DNA or RNA fragments.

10 The nucleic acid fragments of the invention will normally be inserted in suitable vectors to form cloning or expression vectors carrying the nucleic acid fragments of the invention; such novel vectors are also part of the invention. Details concerning the construction of these vectors of the invention 15 will be discussed in context of transformed cells and microorganisms below. The vectors can, depending on purpose and type of application, be in the form of plasmids, phages, cosmids, mini-chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector.

20 Preferred cloning and expression vectors of the invention are capable of autonomous replication, thereby enabling high copy-numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the 25 following features in the 5'→3' direction and in operable linkage: a promoter for driving expression of the nucleic acid fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment of the invention, and a nucleic acid sequence 30 encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the

vector when introduced into a host cell is integrated in the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an animal (i.e. when using the vector in DNA vaccination) it is for security reasons preferred that the vector is not capable of being integrated in the host cell genome; typically, naked DNA or non-integrating viral vectors are used, the choices of which are well-known to the person skilled in the art.

The vectors of the invention are used to transform host cells to produce the analogue of the invention. Such transformed cells, which are also part of the invention, can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recombinant production of the analogues of the invention. Alternatively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid fragment (one single or multiple copies) have been inserted so as to effect secretion or integration into the bacterial membrane or cell-wall of the analogue.

- 20 Preferred transformed cells of the invention are microorganisms such as bacteria (such as the species *Escherichia* [e.g. *E.coli*], *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or *Mycobacterium* [preferably non-pathogenic, e.g. *M. bovis BCG*]), yeasts (such as *Saccharomyces cerevisiae*), and protozoans.
- 25 Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below.
- 30 For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing

the nucleic fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the analogue or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

- 5 When producing the analogue of the invention by means of transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.
- 10 When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the analogue. Preferably, this stable cell line secretes or carries the
- 15 analogue of the invention, thereby facilitating purification thereof.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector
20 ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains
25 genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.

- 30 Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose

promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EP-A-0 036 776). While these are the most commonly used, other microbial promoters have been discussed and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and here the promoter should be capable of driving expression. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available such as *Pichia pastoris*. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the trp₁ gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trp₁ lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate

isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The 5 early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 10 bp sequence extending from the *Hind*III site toward the *Bgl*I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with 15 the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal 20 replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Compositions of the invention

The invention also relates to an immunogenic composition which comprises, as an effective immunogenic agent at least one of 25 the analogues described herein in admixture with a pharmaceutically and immunologically acceptable carrier, vehicle, diluent, or excipient, and optionally an adjuvant, cf also the discussion of these entities in the description of the method of the invention above.

30 Furthermore, the invention also relates to a composition composition for inducing production of antibodies against any

one of the above discussed tumour antigens, the composition comprising

- a nucleic acid fragment or a vector of the invention, and
- a pharmaceutically and immunologically acceptable diluent

5 and/or vehicle and/or carrier and/or excipient and/or adjuvant.

Formulation and other specifics concerning such compositions are discussed in the relevant section regarding nucleic acid immunisation above.

10 EXAMPLE 1

Vaccination against PSM

In the following it will be described how a human autovaccine against PSM can be developed through modification of the molecule by insertion of one or more promiscuous foreign T 15 cell epitopes to reveal a panel of immunogenised PSM molecules.

The constructs will be tested for their ability to induce specific CTL responses against PSM bearing tumour cells.

Furthermore, the constructs will be tested for their ability 20 to induce antibodies which are cross-reactive with the native parts of the PSM molecule. Subsequently, in several *in vitro* assays and *in vivo* animal models the efficacy of the different constructs will be evaluated. The induced antibodies will be tested for their ability to activate complement via the clas- 25 sical pathway and to initiate ADCC via Fc-receptors. Finally, the different molecules will be tested in animal models of human prostate cancer.

Strategy in designing a PSM autovaccine

Briefly, the PSM vaccination plan entails the following experimental tasks

Design and production of a panel of human PSM mutants

- 5 - Cloning of the PSM sequences from human and rat/mouse.
- Mutational work to generate immunogenized hPSM molecules.
- Expression of wild type and immunogenized hPSM molecules in *E. coli* and/or *Pichia pastoris* and/or mammalian cells and/or insect cells (such as the S₂ cell line).
- 10 - Purification, refolding and characterization of the immunogenized hPSM molecules.

DNA vaccination against PSM

- = Generation of hPSM DNA vaccination vectors encoding immunogenized hPSM molecules.
- 15 - Testing of hPSM vaccination vectors in *in vitro* and *in vivo* experiments.

Selection of hPSM candidates

- Immunizations of mice/rabbits.
- ELISA.
- 20 - FACS analysis.
- In case of antibody response: Tumor cell proliferative assay.
- T cell assays.

Testing of the hPSM mutants *in vivo*

- 25 - Solid tumor/metastasis model in mice.

Conceptual study: CTL induction by autovaccination

- Construction of immunogenized mouse/rat PSM corresponding to the selected hPSM candidates (e.g. in the form of DNA vaccines).

- Testing the immune response raised by mouse/rat PSM mutants in *in vitro* assays: Immunochemical assays, ELISA, FACS analysis, cellular assays, complement lysis of PSM bearing cells, ADCC assay, CTL activity assay, Tumor cell proliferative assay, T cell presentation assays.
- Testing of the mPSM mutants *in vivo* in a solid tumor/metastasis model in mice.

Nomenclature of the hPSM constructs

PSM is a type II membrane protein of 750 amino acids, cf. SEQ ID NO: 2 which sets forth the wild-type sequence with the exception that Gly substitutes Trp in position 2 due to the introduction of an NcoI site and a Kozak sequence in SEQ ID NO: 1, where ggt substitutes tgg in positions 4-6. However, native PSM (*i.e.* PSM having a Trp in position 2) has also been used for some human PSM based autovaccine constructs.

In PSM, the extracellular part constitutes the 707 C-terminal amino acids, whereas the cytoplasmic domain is predicted to be 19 amino acids long and the transmembrane part of the protein consists of 24 amino acids (aa 20-43).

As starting point for the constructs, the splice variant PSM' has also been used. Our version of this splice variant has the amino acid sequence corresponding to residues 58-750 in SEQ ID NO: 2. For ease of nomenclature, the regions in PSM' are numbered according to the numbering in PSM (meaning that e.g. region 2 consists of amino acids 87-108 in PSM and amino acids 30-51 in PSM'), cf. also the below discussion of the regions.

All the genetic constructs of human PSM are designated hPSM___. (or hPSM'___. if PSM' is used as a starting point), where the first ___ is the insertion region used for insertion of P2, and the second ___ is the insertion region used for P30.

100

If P2 or P30 is not present in the protein, the number 0 (zero) is designated. The full length wild type hPSM is designated hPSM0.0 and the wild type hPSM lacking the cytoplasmic and transmembrane parts is designated hPSM \div 0.0. The 13 planned 5 immunogenized hPSM genes which all contain one P2 epitope and one P30 epitope will be named hPSM1.1, hPSM6.1, hPSM8.1, hPSM10.1, hPSM1.6, hPSM1.8, hPSM1.10, hPSM1.2, hPSM1.3, hPSM1.5, hPSM2.1, hPSM3.1, hPSM10.3, hPSM6.3, hPSM'10.3, hPSM'6.3, hPSM8.3, hPSM'8.3, and hPSM5.1, cf. details below.

10 In hPSM1.1, both the P2 and the P30 epitopes are inserted in tandem in insertion region no. 1 (the membrane spanning region). Theoretically, this mutant, hPSM1.1, can be considered a very attractive vaccine candidate for induction of antibody production, because the whole extracellular domain of this 15 molecule is intact. For induction of CTL responses using nucleic acid immunization, constructs such as hPSM10.3 and hPSM6.3 are considered useful.

In order to facilitate the cloning and mutagenesis procedures, much of the molecular construction work is done using either 20 the N-terminal (amino acids 1-436) or the C-terminal (amino acids 437-750) part of the hPSM gene as starting material. These two parts of the hPSM gene are designated hPSMI__. and hPSMII__., respectively, where the first __ is the insertion region used for insertion of P2, and the second __ is the 25 insertion region used for P30. Again, if P2 or P30 is not present in the protein, the number 0 (zero) is designated, and the wild types are named hPSMI0.0 and hPSMII0.0, respectively. A special variant of hPSMI0.0 without the cytoplasmic part of hPSM is designated hPSMI \div 0.0.

30 Practically, most mutagenesis work is being done using hPSMI0.0 and hPSMII0.0 as starting material.

The expressed hPSM mutant proteins will be designated PROS___. __, where the first __ is the insertion region used for insertion of P2, and the second __ is the insertion region used for P30. If P2 or P30 is not present in the protein, the 5 number 0 (zero) is designated. The wild type hPSM is designated PROS0.0. PROSII0.0 is the hPSM amino acids 437-750 protein product. HIS tagged proteins are called HIS-PROS___. __. As His tags has been used SEQ ID NO: 21 for expression in yeast and bacteria, whereas SEQ ID NO: 23 has been used for 10 expression in mammalian cells.

Cloning of the human PSM sequence

The LNCaP cell line which originates from a metastatic lesion of human prostatic adenocarcinoma was purchased from the American Type Culture Collection (ATCC). mRNA was isolated 15 from this cell line and reverse transcribed using a standard kit in order to obtain cDNA encoding the human PSM sequence. Using different sets of hPSM specific primers, PCR products encoding PSM(437-750) was obtained and further cloned into pUc19 (plasmid number PMR300) and verified by DNA sequencing. 20 This C-terminal part of wild type PSM is designated hPSM partII (hPSMII0.0).

Similarly, the wild type hPSM partI (hPSM10.0) has been cloned into pUc19 using primers for amplifying partI both with (hPSM10.0) and without (hPSM10.0) the transmembrane+cytoplasmic domains. The clones were control sequenced and hPSM10.0 25 and hPSMII0.0 were fused using ligation at the EcoRI site. The resulting clones of hPSM0.0 (SEQ ID NO: 2) and hPSM10.0 have been subcloned into a number of pro- and eucaryotic expression vectors and again sequence verified. The intracellularly 30 expressed form of human PSM (designated hPSM' - amino acids 58-750 of SEQ ID NO: 2) has also been constructed using the cDNA as starting material. This sequence has also been sub-

cloned into mammalian expression vectors and has been used as starting material for some hPSM autovaccine constructs, e.g. hPSM'10.3 and hPSM'6.3.

Cloning of the rat and mouse PSM sequences

5 Two EST (expressed sequence tag) clones containing murine PSM cDNA sequences (from fetal murine kidney and murine macrophages, respectively) were purchased from American Type Culture Collection (ATCC). Together, these EST's covered the mouse PSM cDNA sequence, and thus both full length mouse PSM (SEQ ID NO: 7 and 8) as well as murine PSM' (SEQ ID NO: 9 and 10) were 10 subcloned into bacterial vectors and mammalian expression vectors. Murine PSM AutoVac construcs have also been made by insertion of P30 into the mouse PSM cDNA.

Expression of wild type hPSM in *E. coli*

15 The C-terminal part (amino acids 437-750) of hPSM, hPSMII0.0, has been cloned into the bacterial expression vector pET28b in order to obtain a product with an N-terminal poly-histidine (HIS) tail which facilitates easy large scale purification and identification with anti-poly-HIS antibodies. The protein 20 product of poly-HIS tagged hPSMII0.0 (protein product designated HIS-PROSII0.0) was expressed in *E. coli*.

The DNA encoding the truncated wild-type human PSM hPSM⁺cyt0.0 has also been expressed from pET28b in the *E. coli* strain BL21(DE3) where the expression product is located in inclusion 25 bodies. SDS-PAGE analysis of bacterial lysate showed a product with the expected migration and Western blotting with rabbit anti-HIS-PROSII0.0 also gave the expected band. Further, N-terminal sequencing of five amino acids of this product eluted from an SDS-PAGE gel gave the expected amino acid sequence.

The wild type hPSM constructs hPSM0.0, hPSM \div 0.0 (as well as two hPSM mutants, hPSM1.1 and hPSM6.1, see below) have been cloned into different *E. coli* expression vectors in order to enable a more efficient expression and some degree of folding 5 of the recombinant proteins in *E. coli*. The chosen expression systems are:

pMCT6 which generates N-terminally His-tagged versions of the expressed recombinant proteins,

10 pGH433 which express the recombinant proteins in connection to a 22 amino acid pelB leader sequence which should direct the protein to the periplasmic space of the *E. coli* bacteria, and

15 pMal-p2 in which recombinant proteins are expressed as C-terminal fusions to maltose binding protein (MBP) containing the natural MBP periplasmic leader sequence. Antibodies against MBP can be used for detection of the fusion proteins and a carbohydrate coupled column can be used for affinity purification of the product.

However, *E. coli* expression experiments of the wild type hPSM proteins from these vectors only showed a fair expression 20 level from pMCT6. The problems of getting periplasmic expression of the wild type hPSM proteins are still not solved at present.

Expression of wild type hPSM in *Pichia pastoris*

Because of the relatively high molecular weight of the PSM 25 protein and its relatively high degree of glycosylation (app. 16% of the molecular weight) and in order to facilitate purification by elimination of the refolding step, it has been decided to implement alternative technology for eukaryotic expression of the recombinant proteins. Several well-charac-

terized eukaryotic expression systems have been evaluated, and for the initial screening of hPSM mutants, the yeast *Pichia pastoris* has been chosen as alternative to *E. coli* expression.

An expression system based on the yeast *Pichia pastoris* has
5 been applied on PSM constructions. The glycosylation pattern
of recombinant proteins expressed in this organism is expected
to resemble the mammalian glycosylation patterns more than
e.g. *Saccharomyces cerevisiae* due to a lesser branched
mannosylation of the recombinant protein. It has been shown
10 that mannose receptor-mediated uptake of antigens by den-
dritic cells results in an approximately 100-fold more effi-
cient presentation to T cells compared to fluid-phase endocy-
tosed uptake. Therefore, mannosylation might play a role for
the antigenicity (and especially the ability to induce CTL
15 responses) of the hPSM mutants and other antigens against
which a CTL response is desirable.

A strain of *Pichia pastoris* as well as two different expres-
sion vectors have been purchased from Invitrogen. The vector
ppICZ α A carries a methanol inducible promoter upstream of the
20 polycloning site, whereas the pGAPZ α A vector express proteins
constitutively. Both vectors encode the α -factor secretion
signal in order to export the recombinant proteins to the
medium. The selection system of these vectors is zeocin resis-
tance. The sequences encoding hPSM0.0, and hPSM \pm 0.0 (as well
25 as one hPSM mutant, hPSM1.1, cf. below) were subcloned into
these vectors (in-frame with a C-terminal c-myc identification
epitope, SEQ ID NO: 27).

Four *Pichia pastoris* strains (X-33, SMD1168, GS115, and KM71)
differing e.g. in their growth requirements were transformed
30 with each of these (linearized) plasmids using electropora-
tion. The transformation procedure was repeated several times
with minor changes in order to obtain a large number of zeocin

resistant clones. Expression of wild type hPSM^{0.0} (as well as hPSM^{1.1}, see below) was obtained in the *Pichia pastoris* system. The expressed products could be detected in Western blotting of lysates of *Pichia pastoris* transformants both 5 using an anti-hPSM monoclonal antibody and an anti-c-myc monoclonal antibody as primary. However, the recombinant products were detected intracellularly.

Expression of wild type hPSM in mammalian cells

An expression system using CHO (chinese hamster ovary) cells 10 will also be implemented for the final testing and production of selected molecules.

So far, CHO cells have been transfected with wild type hPSM and hPSM^{1.1} with/without in frame leader sequences in mammalian expression vector pcDNA3.1. Geneticin resistant cells 15 have been obtained. In COS cells transiently transfected with the same constructs, both hPSM^{0.0} and hPSM^{1.1} was detected in Western Blotting of cell pellets using anti-hPSM monoclonal antibody.

Tissue distribution of hPSM

20 A commercial kit has been purchased in order to determine whether hPSM expression can be detected in various human tissues including prostate, blood and brain. The method is based on a dot blot detection of polyA containing mRNA extracted from 50 different human tissues. Preliminary results 25 do not indicate hyperexpression of hPSM in tissues such as blood or brain. However, after the priority date of the present application, others have demonstrated the presence of PSM in other tissues, cf. above.

Design of the hPSM mutants

When designing the mutational work in PSM, some regions of the protein are very important to preserve in the modified constructs, for example potential and identified T cell epitopes, 5 B cell epitopes and disulfide bridge cysteine residues. Therefore, such "forbidden" regions have been identified within the PSM sequence leaving a limited number of "open" regions of 20 amino acids or more available for exchange with the foreign T helper epitopes P2 and/or P30. Per definition, the transmembrane 10 region is also considered an "open" region since autoantibodies directed against this region are irrelevant and elimination of this sequence is believed to enhance the solubility of the mutated PSM proteins but it cannot be excluded that this region contain important CTL epitopes, hence the 15 preservation of the transmembrane region in e.g. hPSM10.3.

According to our expectation that the autovaccine will induce a CTL response, it would be important to identify and preserve potentially subdominant CTL epitopes in the constructs. Two such epitopes are already known from the literature: 1) the 20 peptide comprising PSM amino acids 4-12 (LLHETDSAV) can be presented on the human MHC class I molecule HLA-A2.1 (*Tjoa 1996*), and 2) the PSM(711-719) (ALFDIESKV) also binds HLA-A2.1 (*ref 25*). We have also searched the PSM amino acid sequence in order to identify primary anchor residues of HLA class I 25 binding motifs as described by Rammensee et al. (*Rammensee, 1995*) for the most abundant HLA class I types (HLA-A1, HLA-A2, HLA-A3, HLA-A23, HLA-A24 and HLA-A28), together constituting 80 % of the HLA-A types of the human population. Likewise, potential HLA-B and HLA-C epitopes have been identified and 30 designated as "forbidden" areas.

Because the initial intention was to use C57/black x SJL F1 hybrid mice in case it was decided to use transgenic mice for

testing the PSM autovaccine constructs, certain potential mouse H-2^b and H-2^s T helper epitopes have been identified and considered "forbidden" regions (Rammensee 1995).

It is also important to preserve known antibody binding regions in the PSM molecule, because they could be important in the induction of specific anti-PSM autoantibodies. Five such regions have already been described: PSM(63-68), PSM(132-137), PSM(482-487) (WO 94/09820), PSM(716-723) and PSM(1-7) (Murphy, 1996). Using the computer based method of Hopp and Woods for prediction of antigenic determinants, five regions are predicted: PSM(63-69), PSM(183-191), PSM(404-414), PSM(479-486) and PSM(716-723) (Hopp 1983), some of these overlapping the experimentally found B cell epitopes. These regions will also be preserved in the PSM vaccine candidate molecules.

15 The PSM protein contains 4 cysteine residues (amino acid positions 22, 196, 466 and 597) which will be preserved in the immunogenized constructs because of their potential importance in the formation of disulfide bridges.

Based on the above mentioned "forbidden" and "open" regions in the hPSM protein, 10 regions suitable for insertion of foreign T helper epitopes were identified:

Insertion regions in **hPSMI** (from initiation site to EcoRI site, aa 1-437):

Region 1: aa 16-52 in PSM (4 aa preceding TM, TM (24 aa) and 25 9 aa after TM = 37 aa)

Region 2: aa 87-108 in PSM, aa 30-51 in PSM' (22 aa)

Region 3: aa 210-230 in PSM, aa 153-173 in PSM' (21 aa)

Region 4: aa 269-289 in PSM, aa 212-232 in PSM' (21 aa)

Region 5: aa 298-324 in PSM, aa 241-267 in PSM' (27 aa)

Insertion regions in **hPSMII** (from EcoRI site to termination site, aa 437-750):

5 Region 6: aa 442-465 in PSM, aa 385-408 in PSM' (24 aa)

Region 7: aa 488-514 in PSM, aa 431-457 in PSM' (27 aa)

Region 8: aa 598-630 in PSM, aa 541-573 in PSM' (33 aa)

Region 9: aa 643-662 in PSM, aa 586-605 in PSM' (20 aa)

Region 10: aa 672-699 in PSM, aa 615-642 in PSM' (28 aa)

10 The insertion regions as well as the "forbidden" regions are represented graphically in Fig. 4.

A number of different immunogenized PSM constructs will be made by substitution of a segment of amino acids from two of the above listed insertion regions with P2 or P30. Each mutant
15 protein will thus contain both P2 and P30, although such constructions are only exemplary - single-mutants are also within the scope of the present invention. Experimentally, the mutations will be made in clones of hPSMI and hPSMII cDNA respectively, and the two mutated parts will subsequently be
20 combined by ligation (at the EcoRI site). The P2 and P30 epitopes have initially been inserted into insertion regions 1, 2, 3, 5, 6, 8 and 10 in order to create the mutants.

The sequences of P2 and P30 are:

P2: QYIKANSKFIGITEL (SEQ ID NO: 12, 15 aa), in this case encoded by the nucleotide sequence cag tac atc aaa gct aac tcc aaa ttc atc ggt atc acc **gag ctg** (SEQ ID NO: 11, 45 nucleotides), where the sequence in boldface is a *SacI* site. Other 5 codon choices may occur, depending on choice of cloning vector and expression system

P30: FNNFTVSFWLRVPKVSASHLE (SEQ ID NO: 14, 21 aa), in this case encoded by the nucleotide sequence ttc aac aac ttc acc gta **agc ttc** tgg ctg cgt gtt ccg aaa gtt agc gCT AGC cac ctg 10 gaa (SEQ ID NO: 13, 63 nucleotides), where boldface indicates an *Hind*III site, single underlining indicates an *Eco*47III site, capital letters indicates a *Bst*NI site, and double underlining indicates an *Nhe*I site.

The following table summarizes the human PSM constructs used 15 herein:

Construct	P2 position in protein	P30 position in protein
hPSM0.0	÷	÷
hPSM±0.0	÷	÷
hPSM'0.0	÷	÷
20 hPSM1.1	17-31	32-52
hPSM6.1	448-462	21-41
hPSM8.1	606-620	21-41
hPSM10.1	674-688	21-41
hPSM1.6	24-38	443-463
25 hPSM1.8	24-38	607-627
hPSM1.10	24-38	673-693
hPSM1.2	24-38	87-107
hPSM1.3	24-38	210-230
hPSM1.5	24-38	301-321
30 hPSM2.1	91-105	21-41
hPSM3.1	213-227	21-41
hPSM5.1	305-319	21-41

Construct	P2 position in protein	P30 position in protein
hPSM8.0	606-620	÷
hPSM10.0	674-688	÷
hPSM0.1	÷	21-41
hPSM1.0	24-38	÷
5 hPSM6.3	448-462	210-230
hPSM8.3	606-620	210-230
hPSM10.3	674-688	210-230
hPSM' 6.3	391-405	153-173
hPSM' 8.3	549-563	153-173
10 hPSM' 10.3	617-631	153-173

Molecular constructions of the hPSM mutants

Mutations to insert P2 and P30 encoding sequences have been performed using both hPSMI0.0 and hPSMII0.0 as starting material.

15 In order to generate a majority of the hPSM mutants, P2 and P30 were initially inserted in hPSMI0.0 at insertion position 1. The resulting material (hPSMI1.0 and hPSMI0.1, respectively) was subsequently used as starting material for mutagenesis to insert P2 and P30 at positions 2,3 and 5 and for 20 ligation with epitope mutated hPSMII. hPSMI1.0 was constructed using SOE (single overlap extension) PCR and subsequently sequence verified. hPSMI0.1 was constructed using the "Quick Change" technique and subsequently sequence verified.

The P2 epitope was inserted into positions 2, 3 and 5 of 25 hPSMI1.0 using SOE-PCR to create hPSMI1.2, hPSMI1.3 and hPSMI1.5. These constructions were combined with hPSMII0.0 to create hPSM1.2, hPSM1.3 and hPSM1.5.

hPSMI2.1, hPSMI3.1 and hPSMI5.1 were constructed by SOE PCR using hPSM10.1 as starting material. This material has been assembled with hPSMII0.0 by ligation at the EcoRI site in order to create the full length mutants hPSM2.1, hPSM3.1 and 5 hPSM5.1.

The P2 epitope was inserted at three different positions of hPSMII0.0 in order to create hPSMII6.0, hPSMII8.0 and hPSMII10.0 using the "Quick Change" technique, and these clones were subsequently sequence verified.

10 Subsequently, hPSM10.1 was ligated with hPSMII6.0, hPSMII8.0 and hPSMII10.0 to obtain hPSM6.1, hPSM8.1 and hPSM10.1, and the clones were sequence verified.

Insertion of the P30 epitope in hPSMII is presently being done to generate hPSMII0.6, hPSMII0.8 and hPSMII0.10 using SOE PCR.

15 hPSM1.1 was constructed using two two-step PCR mutations followed by ligation in a *Hind*III site within the epitope sequence. The full length construct is sequence verified.

hPSM10.3, hPSM'10.3, and hPSM6.3 have been constructed using SOE-PCR. Several other hPSM variants with both P2 and P30 20 inserted in the extracellular part of hPSM are currently being constructed (hPSM'6.3, hPSM8.3, hPSM'8.3).

In addition to the originally contemplated mutants each containing both P2 and P30, four mutants which only contain a single foreign epitope have been constructed and sequence 25 verified: hPSM1.0, hPSM8.0, hPSM10.0 and hPSM0.1.

Expression of hPSM mutants in *E. coli*

In small-scale experiments, seven hPSM mutants, hPSM1.1, hPSM6.1, hPSM8.1, hPSM10.1, hPSM2.1, hPSM3.1 and hPSM5.1 were expressed from pET28b in the *E. coli* strain BL21(DE3), and 5 IPTG inducible products of the expected size were identified on Coomassie Blue stained SDS-PAGE gels. However, a product of hPSM1.1 was not detectable. The expression levels of these hPSM mutants were very low compared to the product of the wild type construct hPSM0.0. At this point, a fair expression 10 level of the hPSM mutants using the pET system in *E. coli* seems impossible, and the use of other *E. coli* expression systems and/or other host organisms is thus necessary.

As mentioned above, hPSM6.1 and hPSM1.1 have been subcloned into different *E. coli* expression vectors in order to generate

15 - N-terminally His-tagged versions of the expressed recombi-
nant proteins using vector pMCT6,
- versions of the proteins expressed with the pelB leader
sequence which directs the protein to the periplasmic space
of the *E. coli* bacteria using vector pGH433, and
20 - versions of the recombinant proteins expressed as a C-
terminal fusion protein to maltose binding protein (MBP)
using vector pMal-p2.

So far, a sufficient expression level from any of these constructs has not been obtained.

25 Since hPSM0.0 is fairly expressed in *E. coli* while a similar expression level of full length hPSM0.0 or the hPSM mutants has not been observed, it is possible that presence of the cytoplasmic part of the hPSM molecule can somehow "inhibit" the expression of the full-length hPSM constructs in *E. coli*.
30 To test this hypothesis, we initially made two genetic con-

structs of hPSM1.1 and hPSM6.1 without cytoplasmic domains. However, in *E.coli* expression experiments there were only weak expression of these \div cyt gene products.

Expression of hPSM mutants in *Pichia pastoris*

5 In order to express the hPSM1.1 mutant protein from the yeast *Pichia pastoris*, the hPSM1.1 sequence has been subcloned (in-frame with a C-terminal c-myc identification epitope, SEQ ID NO: 27) into the two different expression vectors pPICZ α A and pGAPZ α A, and the sequences have been verified. hPSM1.1 expression (as well as hPSM \div 0.0, see above) was detected
10 intracellularly in the *Pichia pastoris* transformants.

Expression of hPSM mutants in mammalian cells

As mentioned above, hPSM1.1 has been subcloned into the mammalian expression vectors pcDNA3.1(+) and pZeoSV2 and these
15 constructs (and others) could be used for expression in e.g. CHO cells. Transient expression of hPSM1.1 as well as hPSM0.0 has been obtained in COS cells as verified by Western blotting.

DNA vaccination

20 DNA vaccination would, if effective, be very well suited for the PSM autovaccine - especially because this administration form has been shown to promote both CTL mediated immune reactions and antibody production. Therefore, it was the intention to perform a parallel study with the aim of investigating the
25 effect of DNA-vaccination of mice with appropriate vectors encoding immunogenized mouse ubiquitin and/or mouse TNF α . DNA vaccination with hPSM (and/or mutants) encoding naked DNA will also be done.

Feasibility study using immunogenized ubiquitin for DNA vaccination

A feasibility study stating the effect of DNA vaccination with an immunogenized self protein was performed using ubiquitin 5 with an inserted T helper epitope from ovalbumin (UbiOVA) as a model protein. Sequences encoding UbiOVA as well as wild type ubiquitin were subcloned into the mammalian expression vector pcDNA3.1(-).

Groups of 5 BALB/c mice were immunized with 40 µg pcDNA-UbiOVA 10 or pcDNA-ubiquitin construct either intramuscularly in the quadriceps or intradermally. An control control group of received UbiOVA protein in complete Freunds adjuvant. Three and six weeks later, the immunizations were repeated with the only difference that the UbiOVA protein was emulsified in 15 incomplete Freunds adjuvant.

The mice were bled regularly and the anti-ubiquitin antibody titers were determined. In the DNA vaccinated UbiOVA groups, only very weak anti-ubiquitin antibody titers were obtained. Subsequently, all groups were boosted with UbiOVA protein in 20 incomplete Freunds adjuvant and bled in order to determine whether DNA vaccination with UbiOVA (and not ubiquitin) could potentiate the antibody response towards UbiOVA protein. The results of this experiment showed that there was no significant difference between the UbiOVA groups and the control 25 groups, all mice developed strong anti-ubiquitin antibodies upon this single UbiOVA/FIA boost.

DNA vaccination using hPSM constructs

Currently, various DNA vaccination experiments are ongoing using hPSM constructs. Various human PSM wildtype and AutoVac 30 constructs (such as e.g. hPSM0.0, hPSM \pm 0.0, hPSM'0.0, hPSM1.1,

hPSM10.3) have been subcloned into DNA vaccination vectors (such as pcDNA3.1(+), pcDNA3.1(-), pVAX and pZeoSV2). In some of the constructions, different leader sequences (such as the CD11a, tPA, and IL-5 leader sequences; SEQ ID NOS: 29, 25, and 5 31, respectively) have been included directly N-terminally and in-frame to allow secretion of the expressed hPSM proteins *in vivo*. All the constructions in DNA vaccination vectors have been verified by DNA sequencing and *in vitro* translation.

Mice of different strains (such as Balb/cA, Balb/cJ, DBA/2 and 10 A/J) have been injected with the above described hPSM DNA vaccines either intradermally or intramuscularly and boosted several times using the same constructs.

Serum samples have been obtained during the immunisation period and stored at -20°C. These samples will be analysed for 15 presence of antibodies reactive with wild type hPSM.

Also, assays to monitor CTL and T helper proliferative responses in these mice are being established.

Preliminary results suggest that induction of both CTL as well as antibody responses against PSM can be accomplished.

20 **Purification/characterization of HIS-tagged hPSM(437-750)
(HIS-PROSII0.0)**

HIS-tagged wild type hPSMII (HIS-PROSII0.0) was expressed from pET28b, and solubilized inclusion bodies were applied to a gel filtration FPLC column and eluted in a buffer containing 8 M 25 urea. Fractions predominantly containing hPSMII were subjected to various refolding conditions to optimize the procedure. Solubilized product dialyzed against a Tris buffer was estimated to be more than 90 % pure using silver-stained SDS-PAGE.

Rabbits were immunized with the purified HIS-PROSII0.0 in order to use the resulting antiserum for later detection and possibly affinity purification of the hPSM mutants.

Purification/characterization of soluble hPSM (PROS÷0.0)

5 Wild type hPSM lacking the cytoplasmic and transmembrane parts, PROS÷0.0, has been expressed in the *E. coli* strain BL21(DE3) upon induction with IPTG and could be detected in inclusion bodies. SDS-PAGE of this bacterial lysate followed by Western blotting with rabbit anti-HIS-PROSII0.0 showed a
10 product with the expected migration. N-terminal sequencing of the first five amino acids of this product eluted from an SDS-PAGE gel showed the expected sequence corresponding to human PSM. The product was subjected to a large series of solubilization and refolding experiments. A product which stay
15 in solution can be obtained in a Tris buffer without denaturant or reductant, but SDS-PAGE analysis reveals that the material probably forms large aggregates. Mice and rabbits have been immunized with this material in order to get antibody against hPSM e.g. for analytical purposes - the antisera
20 did not react with LNCap hPSM.

A batch of washed *E. coli* inclusion bodies of PROS÷0.0 has been prepared for immunization of rabbits to generate a polyclonal antiserum against PSM. Approximately 50% of the protein content in the wet pelleted material contained was
25 PROS÷0.0. The antisera did not react with LNCap hPSM in Western blotting.

Preparation of KLH-conjugated hPSM peptides for immunization

Three 15-mer peptides were synthesized in order to make an immunogenic conjugate of known hPSM B cell epitopes with an
30 immunological carrier molecule to obtain a polyclonal anti-

serum which is able to recognize hPSM. These peptides contain the PSM B cell epitope plus 5-6 flanking PSM amino acids in each end.

The peptides were made by automatic synthesis, HPLC purified
5 and control-sequenced using Edman degradation.

A chemically linked conjugate was prepared by cross-linking the B cell epitope containing hPSM peptides KLH using a standard 1-step procedure with glutaraldehyde as the cross-linking
10 agent.

Synthesis of P2 and P30 peptides with flanking hPSM sequences

Six peptides have been designed which correspond to the P2 and P30 epitopes with 5 flanking hPSM amino acids in each end. The flanking amino acids correspond to the epitope insertion sites
15 6, 8 and 10. The peptides will be used in e.g. T cell proliferation assays, but also for other purposes such as ELISA or other *in vitro* assays. The peptide sequences are:

PSMpep007	P2 inserted in hPSM insertion position 6 <u>QERGVQYIKANSKFIGITELRVDCT</u> (SEQ ID NO: 15)
20 PSMpep008	P2 inserted in hPSM insertion position 8 <u>AVVLROYIKANSKFIGITELEMKY</u> (SEQ ID NO: 16)
PSMpep009	P2 inserted in hPSM insertion position 10 <u>MFLEROYIKANSKFIGITELHVIYA</u> (SEQ ID NO: 17)
PSMpep010 25	P30 inserted in hPSM insertion position 6 <u>NSRLLFNNFTVSFWLRVPKVSASHLEVDCTP</u> (SEQ ID NO: 18)
PSMpep011	P30 inserted in hPSM insertion position 8 <u>VVLRKFNNFTVSFWLRVPKVSASHLESFDSDL</u> (SEQ ID NO: 19)

PSMpep012 P30 inserted in hPSM insertion position 10
LMFLEFNNFTVSFWLRVPKVSASHLEPSSHN (SEQ ID NO: 20)

The P2 and P30 epitopes are underlined. The peptides were made by automatic synthesis and subjected to the process of HPLC
5 purification followed by control-sequencing using Edman degradation.

Immunogenicity assays

Different experimental setups have been initiated in order to produce materials and establish immunogenicity assays for the
10 future testing of and selection between the mutated PSM constructs.

Generation of polyclonal rabbit anti-HIS-PROSII0.0 and anti-KLH-PSM-peptide conjugate antisera

15 Two rabbits were immunized with purified HIS-PROSII0.0, the HIS-tagged C-terminal part of the hPSM protein (amino acids 437-750) emulsified 1:1 with complete Freunds adjuvant and boosted twice (at days 28 and 55) with the same antigen emulsified in incomplete Freunds adjuvant.

20 Two rabbits were immunized with a cocktail consisting of the KLH-PSM peptide conjugate plus each of the three free peptides. These three peptides each contain a previously defined B cell epitope of hPSM. The cocktail was emulsified 1:1 with complete Freunds adjuvant. The rabbits were boosted twice (at
25 days 28 and 55) with the same antigen emulsified in incomplete Freunds adjuvant.

Cross-reactivity between anti-HIS-PROSII0.0 and PSMpep005 and cross-reactivity between anti-KLH-PSM peptide conjugate plus peptides and HIS-PROSII0.0 was demonstrated in ELISA assays.

The anti-HIS-PROSII0.0 antibody has the ability to recognize native hPSM in lysates of LNCaP cells in Western blotting.

Immunization of mice with retrovirally expressed hPSM0.0

At this stage of the PSM project, a serious obstacle is still
5 the lack of antibodies which are able to recognize correctly
folded native hPSM. Therefore, an immunization experiment
using retrovirally expressed hPSM0.0 was performed.

Six groups of Balb/c mice were immunized with either: 1)
mitomycin C treated BALB/c fibrosarcoma cells (79.24.H8)
10 transduced with hPSM0.0 cDNA (CMV-Koz-hPSM), 2) mitomycin C
treated BALB/c fibrosarcoma cells (79.24.H8), transduced with
empty vector (CMVBipep), 3) packaging cells (BOSC) transfected
with hPSM0.0 cDNA (CMV-Koz-hPSM), 4) packaging cells (BOSC)
transfected with empty vector (CMVBipep), 5) retrovirus stock
15 expressing hPSM0.0 cDNA (CMV-Koz-hPSM) or 6) retrovirus stock,
empty vector (CMVBipep).

At several time points, the mice were bled and the sera ob-
tained tested for reactivity in ELISA for reactivity against
HIS-PROSII0.0. Unfortunately, none of the mice developed
20 antibodies able to specifically recognize the HIS-PROSII0.0
preparation.

Establishment of an anti-hPSM ELISA

Purified HIS-PROSII0.0 was used for coating polystyrene micro-
titre plates (Maxisorp) for the purpose of establishing an
25 ELISA assay for testing e.g. hybridoma supernatants or mouse
and rabbit antisera. Sera from BALB/c mice immunized with the
same preparation of HIS-PROSII0.0 were reactive with the
immobilized HIS-PROSII0.0 at 0.5 µg per well using horse

120

radish peroxidase labelled rabbit anti-mouse Ig as secondary antibody.

As mentioned above, the ability of an antiserum raised in rabbits against KLH-PSMpep004-PSMpep005-PSMpep006 conjugate 5 mixed with the free peptides to react with immobilized HIS-PROSII0.0 was demonstrated using this ELISA assay.

Using AquaBind® microtitre plates (cf. the disclosure in WO 94/03530 describing *i.a.* microtitre surfaces coated with tresyl-activated dextran which are now marketed under the 10 registered trademark AquaBind), an ELISA using immobilized PSM peptides (PSMpep004, PSMpep005 and PSMpep006) was established. AquaBind® plates coated with these peptides could detect a rabbit antiserum raised against the same preparation of antigen. As mentioned above, rabbit anti-HIS-PROSII0.0 could be 15 detected on AquaBind® plates coated with PSMpep005.

Establishment of an anti-hPSM Western blot using LNCaP cells and monoclonal antibody 7E11C5

7E11C5 B cell hybridomas which secrete mouse IgG2a monoclonal antibody against an intracellular epitope of human PSM was 20 purchased from ATCC. Culture supernatant from approximately 90% dead cells was collected and used in Western blotting for detection of human PSM in both membrane enriched preparations of LNCaP cells as well as in LNCaP cell lysates. This antibody was purified using protein G columns, and its reactivity with 25 LNCaP in Western blotting verified.

Establishment of a FACS method to detect hPSM on LNCaP cells

We have established two mutually independent FACS methods to detect hPSM on LNCaP cells. Several problems are being addressed: The LNCaP cells grow very slow and in irregular

clumps, and therefore the method to prepare single cell suspensions should be optimized. Secondly, the epitope recognized by the mAb 7E11C5 is in the literature defined to be in the cytoplasmic domain of hPSM. Therefore, the method to fix and 5 permeabilize the cells has been developed. For this purpose, protein G purified 7E11C5 antibody has been FITC conjugated and can thus be used without secondary antibody in FACS analysis.

Also, a FACS method using the anti-hPSM monoclonal antibody 10 J591 which recognizes an epitope on the extracellular part of hPSM, has been established. The antibody was obtained from BZL Biologicals and FITC conjugated and subsequently used for FACS analysis and sortings of e.g. LNCaP cells and hPSM transfectants (see below).

15 Establishing a cytotoxicity assay

A method to purify dendritic cells from mouse bone marrow has been implemented. Using model proteins, immunization of mice with dendritic cells pulsed with model class I peptides and protein has been optimized. Also, mice have been immunized 20 with a model protein (β -galactosidase) formulated in the form of ISCOMS. T-cells purified from immunized mice have been *in vitro* restimulated with different forms of the corresponding antigens. The ability of these restimulated CTLs to lyse different kinds of target cells (including pulsed dendritic 25 cells as well as transfectants expressing retrovirally expressed cytosolic class I peptide) was subsequently measured. Two different *in vitro* assays measuring CTL activity have been established, using either chromium release or and DNA fragmentation (JAM method) as measures of cytotoxicity. Very nice 30 results were obtained with the β -galactosidase model protein and with various combinations of MHC class I and class II binding model peptides.

Establishment of tools to study breaking of autotolerance towards mouse PSM in mice.

It is the intention to study whether autotolerance to mouse PSM can be broken in mice by immunisation and/or DNA vaccination against murine PSM using murine PSM AutoVac molecules.

As mentioned above, cDNA encoding murine PSM (mPSM) has been obtained and DNA sequenced. Four mPSM variant molecules are being generated by insertion of P30 at well-defined sites in either full length mPSM or mPSM'. The constructs are as follows:

	mPSM amino acids substituted with P30	Length of molecule (no. of amino acids)
mPSM0.0	÷	752
mPSM'0.0	÷	694
mPSMX	255-271 (of SEQ ID NO: 8)	756
mPSMY	689-700 (of SEQ ID NO: 8)	760
15 mPSM'X	197-213 (of SEQ ID NO: 10)	698
mPSM'Y	631-642 (of SEQ ID NO: 10)	702

Initially, the mPSM wild type and analogue molecules are subcloned into DNA vaccination vectors and used for DNA vaccination of mice.

20 It is the intention to analyse immune responses such as CTL responses and tumor elimination in the mice. For this, murine tumor cell lines will be transfected with wild type murine PSM (fused in-frame with an identification tag, e.g. the c-myc epitope, SEQ ID NO: 27, for detection purposes).

in vivo PSM tumor models**Mouse T cell proliferation assays with P2 and P30**

A series of T cell proliferation experiments has been conducted in order to establish the T cell immunogenicity of P2 and P30 peptides in various mouse strains (BALB/cA (H-2^d), C3H/Hen (H-2^k), DBA/1 (H-2^q) and C57BL/6 (H-2^b)). It is well known that these epitopes are promiscuous in humans, but the T cell promiscuity also needed to be confirmed in mice using M&Es experimental setup. It was thus shown that P30 is T cell immunogenic in the BALB/cA and C57BL/6 strains whereas neither P2 or P30 were shown to be T cell immunogenic in the C3H/Hen strain. In DBA/1, T cells could be raised against P2.

Generation of hPSM expressing mouse tumor cells

For the use of a hPSM specific tumor model in mice as well as for the use in tumor cell proliferative assays, a panel of hPSM expressing mouse tumor cells are being established.

One approach is to generate these cell lines by transducing the murine tumor cell lines with retroviral vectors encoding the full-length wild type hPSM0.0 cDNA.

Three different constructs encoding full length wild type cDNA encoding human PSM inserted into the polycloning site of the retroviral vector CMVBipep was constructed, two of these containing a short Kozak sequence upstream of the start codon.

These constructs were transduced into three different mouse tumor cell lines: P815 (mastocytoma, H-2^d), B16-F10 (melanoma, H-2^b) and 79.24.H8 (fibrosarcoma, H-2^d) using the BOSC packaging cell line. Geneticin resistant clones have been obtained for all three cell types, and it was verified in PCR analysis

on genomic DNA template that the retroviral constructs were integrated in the host cells. It has not yet been possible to detect an expressed PSM gene product in Western blot or FACS analysis using the 7E11C5 monoclonal antibody.

5 Two stable mouse tumor cell lines harboring membrane bound wild type human PSM have been established by transfection. This was done using hPSM0.0 cDNA subcloned in the mammalian expression vector pcDNA3.1(+) under the control of the CMV promoter and containing a Kozak sequence upstream of the start 10 codon.

The resulting plasmid was transfected into two different mouse tumor cell lines: 79.24.H8 (fibrosarcoma, Balb/c derived) and Sa1N (fibrosarcoma, A/J derived). Geneticin resistant cultures were obtained and subjected to Western blotting and FACS 15 analysis using the J591 and 7E11C5 anti-hPSM monoclonal antibodies. Using the J591 antibody, the cells were FACS sorted several rounds until a hPSM positive population was obtained. hPSM expression was again verified by intracellular FACS staining using the 7E11C5 antibody. It was also checked by 20 FACS analysis that the MHC class I expression levels were the same level as the levels of the parental cell lines.

Cultures of 79.24.H8 and Sa1N cell lines expressing hPSM were cloned by limiting dilution. Several clones were obtained and tested for different hPSM expression levels by FACS analysis 25 using the anti-hPSM monoclonal antibody J591.

79.24.H8 cells expressing hPSM were transfected with the gene encoding B7.1 for use in e.g. *in vitro* assays to monitor hPSM specific CTL responses and/or interferon-gamma release. The cells were FACS sorted one time using an anti-B7.1 antiserum.

Establishment of a hPSM specific tumor model in mice

It has been decided to establish at least two *in vivo* tumor models in immune competent mice in order to determine the anti-tumor effect of antibodies raised in mice against the 5 immunogenized hPSM molecules. This will hopefully be done by injecting syngeneic mouse tumor cell lines modified to express wild type hPSM on the surface membrane. Cells which form solid tumors and/or cells which are known to metastasize will be used. Cell lines which can be implanted in syngeneic mice 10 without being rejected due to the presence of the foreign hPSM molecule will be used in the model. The ability of the hPSM vaccines to eliminate such tumor cells will be used for the selection of the hPSM vaccine candidates.

To evaluate the growth of Sa1N cells transfected with the full 15 length human PSM, different doses (2×10^6 and 5×10^6) of the hPSM transfected Sa1N cells (S-PSM, sorted 5 times) were injected subcutaneously at the lower right flank of groups of A/J mice. However, solid tumors did not establish. Subsequently, three clones of S-PSM cells with different expression level of hPSM 20 were injected subcutaneously in 3 groups of A/J mice at a dose of 10^7 cells/mouse. The sizes of the established tumors were measured with a caliber measuring two different diameters which were multiplied to give the tumor size in mm^2 . These values were compared for the three groups. Within 3-6 days, 25 all mice developed a solid tumor-like structure which disappeared again approximately by day 15. This is likely to be due to the presence of human PSM on the tumor cell surfaces, although it has not yet been verified. Sa1N cells transfected only with the pcDNA3.1 vector continued to grow as solid 30 tumors in mice.

A similar picture was observed in mice injected with 10^6 , 5×10^6 , or 10^7 79.24.H8 cells transfected with hPSM and sorted

several rounds for hPSM expression. These cells (termed 79-PSM) also did not establish as tumors in Balb/c nor DBA/2 mice. However, when a clone of hPSM transfected 79.24.H8 cells, 79-PSM.3, was injected into Balb/c or DBA/2 mice, the 5 mice developed solid tumor-like structures which disappeared again by day 10-20. Vector-transfected 79.24.H8 continued to grow in Balb/c mice.

It still remains to be evaluated if these "tumors" are treatable, or if a better tumor model can be established based on 10 the described S-PSM and 79-PSM cell lines and clones.

Conclusions

In the molecular construction work we have succeeded in cloning of the human PSM gene and obtaining the mouse PSM cDNA. An array of fully sequenced immunogenized hPSM autovaccine 15 constructs have been produced. The hPSM mutants as well as different wild type hPSM molecules have been expressed in *E. coli*, and it was found and verified that the expression level in *E. coli* is very low. Polyclonal antibodies against the C-terminal half of hPSM have been induced in rabbits. Efforts 20 have been made in order to implement different expression tags (His-tag and maltose binding protein fusion) as well as expression systems alternative to *E. coli* inclusion bodies. Recombinant wild type and/or autovaccine hPSM has been detected in transfected *Pichia pastoris* and mammalian cells. 25 Useful considerations regarding the DNA vaccine technology have been made, and a preliminary feasibility study was performed. DNA vaccination experiments with hPSM autovaccine molecules are ongoing and show promising preliminary results. Different *in vitro* assays useful for testing of and selection 30 between the mutated PSM constructs is established, including immunochemical assays and FACS analysis. Mouse tumor cells have been stably transfected with full length wild type hPSM

and FACS sorted for hPSM surface expression. Clones of these cell lines have been obtained. *In vivo* xenogenic tumor models in mice is being evaluated using these hPSM bearing syngeneic mouse tumor cells. An array of T cell proliferation assays 5 have been performed in order to select the mouse strains for the tumour models. CTL assays are being optimized, and convincing results with model antigens have been obtained using different immunization methods and assay conditions. Furthermore, tools necessary to study breaking of tolerance to mouse 10 PSM by immunization against mouse PSM autovaccines are being established.

EXAMPLE 2

Production of a Her2 autovaccine

A human autovaccine against Her2 can be developed through 15 modification of the molecule by insertion of one or more promiscuous foreign T cell epitopes to reveal a panel of immunogenised Her2 molecules. These modified proteins will be tested for their ability to induce antibodies which are cross-reactive with the native parts of the Her2 molecule. Subsequently, 20 in several *in vitro* assays and *in vivo* animal models, the efficacy of the different constructs (as may be the case with the DNA vaccination) and modified proteins will be evaluated. The induction of specific CTL responses against Her2 bearing tumour cells will be analysed. Also, the induced 25 antibodies will be tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different modified molecules will be tested in animal models of human breast cancer to examine their effects on the treatment of tumours.

Immunogenic rat and human molecules will be constructed with promiscuous T-cell epitopes at different positions in the molecule.

During vaccination against the entire extracellular domain of Her2 there is a possibility of some degree of cross reaction of the antibodies with other EGFr receptors since some of these receptors are homologous by up to 40-46% in the extracellular domains. Therefore it is planned that the conserved regions of Her2 would be disrupted by inserting foreign T cell epitopes at least in some of the modified proteins (see below for details).

Regions of Her2 that may potentially be CTL or B-cell epitopes are avoided in designing of constructs are seen in Fig. 3. The rationale for using these positions is as follows:

15 The human Her2 sequence can be divided into a number of domains based solely on the primary structure of the protein.

Extracellular (receptor) part:

1-173: Domain I (N-terminal domain of mature polypeptide).

20 174-323: Domain II (Cysteine rich domain, 24 cysteine residues).

324-483: Domain III (ligand binding domain in the homologous EGF receptor).

25 484-623: Domain IV (Cysteine rich domain, 20 cysteine residues).

624-654: Transmembrane domain (TM residues from 654 - 675).

Intracellular (kinase) part:

655-1010: Tyrosine kinase domain (core TK domain from 725 - 992).

1011-1235: C-terminal domain.

5 Selection of sites in the amino acid sequence of HER2 to be displaced by either the P2 or P30 human T helper epitopes has been done considering the following parameters (loosely prioritised):

1. Known and predicted CTL epitopes
- 10 2. Homology to related receptors (EGFR in particular)
3. Conservation of cysteine residues
4. Predicted loop, α -helix and β -sheet structures
5. Potential N-glycosylation sites
6. Prediction of exposed and buried amino acid residues
- 15 7. Domain organisation

The CTL epitopes appear to be clustered in domain I, domain III, the TM domain and in two or three "hot spots" in the TK domain. According to the invention, these should be largely conserved.

20 Regions with a high degree of homology with other receptors are likely to be structurally important for the "overall" tertiary structure of Her2, and hence for antibody recognition, whereas regions with low homology possibly can be exchanged with only local alterations of the structure as the
25 consequence.

Cysteine residues are often involved in intramolecular disulphide bridge formation and are thus crucial for the tertiary structure and should preferably not be changed.

Regions predicted to form α -helix or β -sheet structures should preferably be avoided as insertion points of foreign epitopes, as these regions are probably important for the folding of the protein.

5 Potential N-glycosylation sites should preferably also be conserved because mannosylation of the protein (for example by expression in yeast) is desired, cf. the presence of mannose receptors on APCs.

Regions predicted (by their hydrophobic properties) to be
10 interior in the molecule preferably should be conserved as these could be involved in the folding. In contrast, solvent exposed regions could serve as candidate positions for insertion of the model T_h epitopes P2 and P30.

Finally, the domain organisation of the protein has also been
15 taken into consideration because of its relevance for protein structure and function.

The focus of the strategy has been to conserve the structure of the extracellular part of Her2 as much as possible, because this is the part of the protein which is relevant as target
20 for neutralising antibodies. By contrast, the intracellular part of native membrane bound Her2 on the surface of cancer cells is inaccessible for the humoral immune system.

Hence, only the presence of CTL epitopes gives reason to include this part in a vaccine. It is therefore obvious to
25 place one or more epitopes here. If it turns out that it is impossible to express the full length Her22 molecule in *E. coli* or in yeast, the intracellular part could be truncated after the first CTL epitope "hot spot" (around position 800). Additional CTL epitopes can hereafter be added to the C-terminal end of the truncated molecule.
30

The transmembrane region probably is an independent folding unit and substitution of this with T_H epitopes such as P2 or P30 will probably not affect the HER2 structure and folding. In addition, the TM domain might cause great problems for the 5 expression in yeast and coli and should in any case be substituted. Thus, an epitope should preferably be placed in this domain in all constructions (perhaps leaving it intact in 1 construction as it contains several CTL epitopes and because it is somehow involved in transmission of signals upon ligand 10 binding).

The extracellular domain could principally be kept intact by placing P2 and P30 in the intracellular and transmembrane domains, thereby maximising the number of potential B-cell epitopes and interfering as little as possible with the structure. However, the high degree of homology to EGFR and Her-3 and Her-4 make a risk for cross reactivity to these receptors which may (or may not) be undesirable. In addition, some 15 monoclonal antibodies have been described which function as agonists for the receptor (perhaps by stimulating heterodimerisation or ligand binding) and increase tumour size, *in vivo*. Positions in the extracellular domain have therefore been selected which thereby hopefully will reduce these risks.

This selection has involved all of the before mentioned parameters and has been based on two different assumptions: (i) Insertion in non-conserved (with respect to related receptors) regions will help to maintain the tertiary structure and might reduce unwanted activation by antibodies. (ii) Insertion in the well conserved regions can alter the structure, but might 30 at the same time reduce the risk of cross reactivity by destroying the most related sequences. Both assumptions are speculative, but as it is very difficult to predict the effect

of placing an epitope in any position of the protein some of these positions have been included in the constructions.

It has been speculated that it could be an advantage to remove the two cysteine rich domains completely. These are predicted 5 to form solvent exposed loop structures and could form independent folding units perhaps involved in dimerisation (as indicated by the many cysteines that could serve to keep a rigid structure necessary to form a dimerisation domain). Deleting these structures might therefore eliminate the risk 10 of activation by antibodies as well as reduce the number of cross reacting antibodies as these domains are the most well conserved of the extracellular part of the protein. In addition, such cysteine rich domains could be problematic to produce in *E. coli* or yeast cells.

15 The details of constructs are as follows using the P2 and P30 epitopes as non-limiting examples: the P2 epitope will primarily be placed in the extracellular domain of Her2 in combination with the P30 epitope substituting part of or all of the membrane spanning region. The P2 epitope will be placed in 20 regions based on the criteria discussed above. The preferred constructs have the following structures:

Construct name	Position of P2	Position of P30	Length
hHER2MA2-1A	59-73	632-652	795
hHER2MA2-2A	103-117	632-652	795
25 hHER2MA2-3A	149-163	632-652	795
hHER2MA2-4A	210-224	632-652	795
hHER2MA2-5A	250-264	632-652	795
hHER2MA2-6A	325-339	632-652	795
hHER2MA2-7A	369-383	632-652	795
30 hHER2MA2-8A	465-479	632-652	795
hHER2MA2-9A	579-593	632-652	795
hHER2MA2-1B	59-73	661-675	795
hHER2MA2-2B	103-117	661-675	795
hHER2MA2-3B	149-163	661-675	795

	Construct name	Position of P2	Position of P30	Length
	hHER2MA2-4B	210-224	661-675	795
	hHER2MA2-5B	250-264	661-675	795
	hHER2MA2-6B	325-339	661-675	795
	hHER2MA2-7B	369-383	661-675	795
5	hHER2MA2-8B	465-479	661-675	795
	hHER2MA2-9B	579-593	661-675	795
	hHER2MA2-1Y	59-73	710-730	795
	hHER2MA2-2Y	103-117	710-730	795
	hHER2MA2-3Y	149-163	710-730	795
10	hHER2MA2-4Y	210-224	710-730	795
	hHER2MA2-5Y	250-264	710-730	795
	hHER2MA2-6Y	325-339	710-730	795
	hHER2MA2-7Y	369-383	710-730	795
	hHER2MA2-8Y	465-479	710-730	795
15	hHER2MA2-9Y	579-593	710-730	795
	hHER2MA2-Z	695-709	710-730	795
	hHER2MA2-C	653-667	632-652	795
	hHER2MA2-BX	695-709	661-675	795
	hHER2MA2-AX	695-709	632-652	795
20	hHER2MA2-4E	210-224	5-25	795
	hHER2MA2-6E	325-339	5-25	795
	hHER2MA2-8E	465-479	5-25	795
	hHER2MA5-4D	210-224	632-652*	666
	hHER2MA5-6D	325-339	632-652*	666
25	hHER2MA5-8D	465-479	632-652*	666
	hHER2MA6-C	653-667	632-652	702

Position of the epitope indicates the first and the last amino acid position of the epitope relative to the start point of mature Her2. Length is the length in amino acids of the complete construct. In all constructs except those where position is indicated with *, the epitope substitutes an amino acid stretch of the same length as the epitope. "*" Indicates that the epitope is inserted rather than substituted into Her2. All constructs listed above are therefore truncates of mature Her2, where the omitted part is from the C-terminus.

Most of the constructions exist in different versions, e.g. in pcDNA3.1+ vector in fusion with the natural HER2 signal pep-

tide sequence, in the vector pMT/BiP/V5-His-A as a fusion with the BiP leader peptide for expression in Drosophila cells and without leader sequence in the pET28b vector for expression in E. coli cells.

5 Below are described the models that are intended for use in the screening and selection of modified Her2 proteins.

1. Induction of antibodies in transgenic rat Her2 mice and in rabbits to rat and human Her2, respectively, will be investigated by conventional ELISA technology after at least 10 three immunisations. Commercially available antibodies to human and rat Her2 will be used as positive controls.
2. These rabbit antibodies will be used to study the putative inhibition of growth of human and transgenic mouse tumour cells overexpressing Her2 in an *in vitro* model.
- 15 3. T cell proliferation of PBL from tetanus immunised patients towards selected human Her2 molecules will be investigated by conventional methods.
4. The ability of modified rat Her2 molecules to induce CTL responses in rat Her2 transgenic mice will be studied using 20 tumours from these mice as targets.
5. It is intended to synthesise a selected set of peptides in the transmembrane region of human Her2 encompassing P2 and P30 epitopes. These peptides will be tested in proliferation of PBL from humans previously immunized with tetanus 25 toxoid to determine whether P2 and P30 epitopes could be efficiently processed out from within the Her2 sequences and presented to T cells.

6. It is quite possible that selected human modified Her2 proteins will be tested to generate neutralising antibodies in a mouse that has been genetically constructed to only expresses human VDJ genes. Such a mouse is available from
5 Abgenix, Fremont, CA, U.S.A. as a collaboration.

Four well-characterised transgenic mouse models for breast cancer that contains rat Her2 oncogene have been described. The first three transgenic mice express activated Her2 oncogene while the fourth model utilises inactivated Her2. All 10 models utilise an MMTV promoter to drive expression in mammary glands.

We have decided to use two transgenic mice models: 1) a more aggressive tumour model described by Muller et al using activated Her2 oncogene (*Muller et al, 1989*) and 2) a less aggressive tumour model in which inactivated Her2 is used to 15 create focal mammary tumours with long latency (*Guy et al, 1992*). Both transgenic mice are purchased from Jackson and/or Charles Rivers Laboratories.

In the initial experiments, these mice are allowed to produce 20 antibodies and CTL responses by immunising and boosting with modified rat Her2 proteins. Incidence of tumours will then be investigated as described by others (*Muller et al, 1989; Guy et al, 1992; Katsumata et al, 1995*). Antibody levels will be measured by an ELISA assay. The CTL activity would be determined by generating target cells expressing rat Her2 as mentioned above.
25

Alternatively, the nude mouse xenograft carcinoma model can be used for passive vaccination experiments. Nude mice can be transplanted with human tumours and inhibition of tumours 30 could be attempted with passive transfer of serum from normal or humanised mice immunised with modified Her2 proteins. While

this would be useful for studying the role of antibody in suppressing tumours, CTL activity cannot be directly measured in this system.

In the second *in vivo* model, tumours in mice would also be
5 generated by transplanting cells lines from tumours of transgenic mice described above. Cell lines generated from these mice would be transferred into relevant mouse strain and localisation established using standard protocols.

Transfer of mouse tumours cells over expressing rat Her2:
10 In this system, cells will be transfected with rat genes and transferred into MHC compatible mice. Inhibition of tumour growth would be achieved by generating anti-Her2 responses.

In these systems; modified Her2 proteins would be used as vaccine in adjuvants to generate antibodies and CTL responses.

15 DNA vaccination has been used successfully in several systems to mount an effective immune response. We are currently investigating means of DNA delivery using modified self proteins. It is our intention to utilise DNA vaccination approach to determine effects of modified Her2 constructs in inhibiting
20 tumours in transgenic mouse models of breast cancer. Similar approach can than possibly be applied in humans for the treatment of this disease.

EXAMPLE 3

Production of an anti-FGF8b vaccine

25 In the following it will be described how a human autovaccine against FGF8b can be developed through modification of the molecule by insertion of one or more promiscuous foreign T

cell epitopes to reveal a panel of immunogenized "FGF8b" molecules. The constructs will be tested for their ability to induce antibodies that are cross-reactive with the authentic parts of the FGF8b molecule. Subsequently, in several *in vitro* assays and *in vivo* animal models the efficacy of the different constructs will be evaluated. The induced antibodies will be tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different molecules will be tested in animal models of human prostate and breast cancers.

Construction of an autovaccine against FGF8b

Due to the complete identity of the murine and human FGF8b polypeptides, all constructs can be used for experiments in both humans and mice.

The promiscuous tetanus toxoid T helper cell epitopes P2 and P30 used with success in the human TNF α vaccine will be inserted into the FGF8b polypeptide. Due to the small size of FGF8b, constructs will be made with one epitope per molecule. Other promiscuous T helper cell epitopes such as the influenza haemagglutinin epitope HA(307-319) and other T-cell epitopes discussed herein could also be considered (O'Sullivan 1991).

4 different immunogenized FGF8b constructs have been made, with the epitopes distributed along the molecule. These four constructs are made on the basis of multiple and pairwise alignments of the FGF family of proteins. A pairwise alignment of FGF2 and FGF8b is used as basis for an analysis of the presumed secondary structure (i.e. beta-sheet distribution) along the FGF8b molecule. The residues that are conserved between FGF2 and FGF8b does not cluster anywhere on the three-dimensional structure, which indicates that there are no particular regions of the molecule that cannot be replaced

without having deleterious effects on the folding capabilities. The amino acid residues in FGF2 that align to the cysteine residues in FGF8b are positioned very close to each other three-dimensionally, indicating that they form a disulfide bond in FGF8b, and that the alignment is correct. The flexibility of the N-terminal part of FGF2 was also considered.

The variant of FGF8b with the P30 epitope in the N-terminal (F30N) was designed on the basis of no-gap alignments of the 10 amino acid residues of the FGF8b protein and the P30 epitope (SEQ ID NO: 14), and scoring the different positions with regard to chemical properties of every amino acid residue. Only the region N-terminally of the predicted beta-barrel structure was considered. In the case of F30N, there are 9 similar out of 21 residues. Using this pseudo-algorithm, the 15 substitutions would be expected to result in minimal overall structural changes. The sequences of the four different constructs, as well as three-dimensional representations of the replaced amino acids are shown in Figure 6.

The variant of FGF8b with the P2 epitope (SEQ ID NO: 12) in the C-terminal (F2C) was initially designed as F30N. There is, however, predicted a good Kd epitope at positions 195-203. Therefore, the P2 epitope is inserted just C-terminal of this epitope. Again, only the region C-terminal of the predicted 25 beta-barrel was considered.

The internal variants of FGF8b (F30I and F2I) were constructed by replacing external loops in the FGF2 structure with the epitopes P2 and P30, respectively, whereby the beta-barrel structural backbone of the FGF structure presumably will 30 remain unchanged.

The immunogenized FGF8b molecules have been expressed in *Eschericia coli*, which facilitates large scale production of the proteins at minimal costs. Although, FGF8b contains two potential N-glycosylation sites (Asn31 and Asn177), bacterially expressed recombinant FGF8b has been shown to be biologically active (MacArthur 1995a, Blunt 1997). In order to facilitate purification and refolding, the FGF8b variants have been produced in a His-tagged version, thereby rendering coupling to a Ni-charged column possible.

Purification of the molecules has been performed utilizing the high positive charge of the protein molecules or the His-tag, and refolding will be performed using standard procedures taking the formation of the disulfide bridge into account.

The four immunogenized molecules have also together with the wild type FGF8b cDNA been inserted into DNA vaccination vectors.

Screening and selection of the modified FGF8b molecules

The four immunogenized FGF8b molecules have been expressed in bacteria and subsequently purified from inclusion bodies. In parallel, the constructs will be used as DNA vaccines. The different constructs will then be compared for their ability to induce various effects, which are desired in the treatment of prostate and breast cancer patients. Such investigations will be performed using several different *in vitro* and *in vivo* assays. Finally, the results of the experiments will form the basis for the ultimate selection of one or two candidates for a FGF8b vaccine in humans.

in vitro models**Analyses in the murine system**

Mice of different haplotypes as well as rabbits will be immunized with the different FGF8b constructs in Complete Freund's Adjuvant and subsequently boosted at least twice with the same antigens emulsified in Incomplete Freunds Adjuvant. Thus, the ability of the different constructs to break B-cell tolerance can be compared. DNA vaccination will be performed on other animals using purified DNA in Complete Freund's Adjuvant /
10 Incomplete Freund's Adjuvant injected intra muscularly with 14 day intervals.

Serum samples will be obtained at several time points during the immunization schedule, and the ability of the different constructs to induce FGF8b specific antibodies will be determined using a conventional ELISA method (Rochon 1994). A commercial polyclonal antiserum, as well as a commercial monoclonal antibody raised against FGF8b (R&D), would be used for positive controls. The FGF8b protein is commercially available (R&D) but will also be produced along with the other FGF8b constructs and subsequently purified/refolded. This product can then be used for coating of plates in a direct ELISA for testing the sera from mice/rabbits immunized with FGF8b variant proteins.
20

A valuable tool for investigating the effects of vaccinating against FGF8b will be a FGF8b dependent cancer cell line. Several FGF8b positive cancer cell lines, e.g. MCF-7 or SC-3, are described in the literature. Such a FGF8b dependent murine cancer cell line will be identified using quantitative RT-PCR, cell proliferation experiments, and STAT-3 phosphorylation assays.
30

The presence of FGF8b ligated to a FGF receptor on the cell surface will be detected with FGF8b specific antibodies in FACS or ELISA analysis. Antibodies directed against several of the different FGF receptors are commercially available (R&D).

- 5 The constructs will be compared with respect to their ability to induce antibodies capable of activating complement lysis of FGF8b producing / bearing cells. This can be detected with one of the mouse tumor cell lines expressing FGF8b described earlier or, alternatively using osmotically FGF8b-loaded
- 10 cells. Sera from normal or transgenic mice (see below) immunized with the human FGF8b constructs will be incubated with the cell line and subsequently incubated with fresh guinea pig complement. Antibody mediated complement lysis of the cells can be detected by standard procedures.
- 15 The ability of the induced antibodies to mediate ADCC can be studied by measuring the ^{51}Cr -release from labeled FGF8b expressing cells. The effector cells will be PBMC from syngeneic mice. For establishing the assay, it may be convenient to use a mouse cell line capable of mediating ADCC (positive for
- 20 Fc(-receptors) as effector cell with an antibody against human FGF8b.

In order to show that the FGF8b candidate vaccines do not somehow promote autoantibody induced tumor growth we will also perform a tumor proliferation assay. Serum samples from immunized mice will be incubated with FGF8b expressing tumor cells. Proliferation of the tumor cells can then be detected by their ability to incorporate ^3H -thymidine, which subsequently is added to the cells.

Since FGF8b is known to induce proliferation of a range of mammalian cells, it will also be necessary to examine the growth promoting effects of the variant proteins. This can be

done using cell proliferation assays as the one used by Marsh 1999.

The biological effect of FGF8b on mammalian cells should be neutralized by the autoantibodies. This can be demonstrated by 5 using recombinant FGF8b and e.g. NIH3T3 in cell proliferation (and morphology changes) studies. Addition of the autoantibodies should abolish the transforming activity of FGF8b.

Immunization protocol

The number of animals that are to enter a FGF8b AutoVac immunization experiment must depend on the expected penetrance of 10 the disease in the model, and thus, the numbers needed to obtain statistically significant information. The immunization protocol must be based on the experience we have from the TNFa AutoVac project. Various immunization protocols have been used 15 for immunizing mice with the various TNFa analogs for specific purposes, but most experiments were performed using the following protocol:

1. The mice should be individually marked either by earmarks or with transponders, 10 animals in each cage. Presumably, 20 males and females must be evaluated separately, but in any case, we will not have both sexes in the same cage. The animals should be left to rest at least 3 days after transport and marking.
2. Antigen 1 mg/ml in PBS buffer was emulsified with an equal 25 volume Freunds complete antigen (CFA) (Difco or Sigma). The emulsion is checked by placing a drop of the emulsion on a water surface and it is observed whether the drop holds together or disperses. Mixing is maintained until the drop does not disperse.

3. The standard immunization dose is 100 µg antigen in a 100 µl volume + 100 µl of adjuvant. Thus, the total immunization volume is 200 µl, administered s.c. (sub cutaneously) over the back of the animal.

5 4. Boostings are performed 2-3 weeks after the primary immunization, and subsequently at 2-3 week intervals. The boosting/immunization material is prepared and administered exactly as the immunization material, but Freunds incomplete adjuvant is used. Probably three boosts will induce the maximal titer.

10 Thus, the highest titers will be obtained 6-9 weeks after the first immunization.

5. Bleedings are orbital bleeds of 50-100 µl usually taken before the first immunization and one week after each boosting. Tail bleeds can also be used, and 10-20 µl can be sufficient for titre determinations.

The initiation point of the immunization program will depend on the development of the disease, and the strategy we want to adopt. Initially, we suggest that it is attempted to generate the maximal immunity as soon as possible, but it is difficult 20 to start immunizations sooner than at approx. 5 weeks of age. Hereafter, high titres should be maintained by boosting at 6-8 week intervals, after the three initial boosts. There is a potential problem if the FGF8b is necessary for the normal development of the young mouse, and therefore one could argue 25 in favor of starting the immunizations later in the adult mouse.

Analyses in the human system

In the selection between the different FGF8b constructs the ability of human antigen presenting cells to present the 30 inserted immunogenic T cell epitopes to human T cells will be

investigated. This will be done by using the same *in vitro* processing assays for P2 and P30 presentation that were used for the TNFa vaccine. Human T cell lines, which are specific for P2 and P30, will be established from donors vaccinated
5 against tetanus. Antigen presenting cells (PBMCs) from the same donors will be incubated with the different constructs and T cell lines will be added. The level of presentation of the inserted T cell epitopes can then be compared by measuring the stimulation of the T cell lines.

10 ***in vivo* animal models**

At least three different systems can be used to monitor whether the induced FGF8b antibodies are capable of controlling a FGF8b dependent *in vivo* effect.

Mice will be transplanted with murine FGF8 expressing tumor
15 cells, and inhibition of tumor progression will be assayed with autovaccination using the modified FGF8b proteins or FGF8b DNA vaccines. The ideal system involves the use of cells isolated from murine tumors. Alternatively, we will use other murine cell lines (e.g. Balb/3T3) stably transfected with the
20 FGF8b cDNA in an expression vector.

The mouse xenograft carcinoma model will be used for passive vaccination experiments. Nude mice will be transplanted with human tumors, and inhibition of tumors would be attempted with transfer of serum from normal or humanized mice immunized with
25 modified FGF8b proteins or FGF8b DNA vaccines. This would be very useful for studying the ability of the raised antibodies to suppress tumors.

Another approach to achieve proof of concept will involve the use of mice transgenic for FGF8b. These mice, that are carrying the FGF8b cDNA under control of the very specific mouse

mammary tumor virus (MMTV) promoter, are shown to spontaneously develop FGF8b expressing mammary tumors (Coombes personal communication). Autovaccination of these mice with the FGF8b variant proteins or FGF8b DNA vaccines would enable us
5 to show if the autovaccine will enable the mice to suppress or reject the tumors.

A possible approach to obtain proof of concept would be to use the Wnt-1 transgenic mice (MacArthur 1995c). Induction of breast cancers by MMTV virus is reported to activate FGF8
10 expression in more than half of the mice developing tumors. FGF8b-dependency of the tumors, could be established if our autovaccine(s) could suppress the incidence or growth rate of the tumors.

The fact that transgenic mice often show non-physiological
15 immunological tolerance patterns will most likely not affect this project since the FGF8b polypeptides are identical for human and mouse.

When, a beneficial effect of the FGF8b immunizations eventually has been demonstrated in the mouse model and suitable
20 human vaccine candidates have been selected it will be possible to perfom a limited number of toxicology studies. Subsequently, to obtain a final proof of concept, vaccine studies on breast, and prostate cancer patients can be carried out.

Importantly, if the experiments using *in vivo* models have
25 positive outcome, more mutants will be constructed based on the data available.

EXAMPLE 4

Preparation of MUC-1 analogue

Only one MUC-1 autovac molecule has been made. This comprises, in total, nine mucin repeats each having the sequence SEQ ID NO: 33. The construction starts with three such sequences, followed by a P2 epitope, followed by three more mucin sequences, followed by a P30 epitope, ended by three mucin sequences.

The construction has been made with and without an N-terminal UNI-his tag (SEQ ID NO: 23). Both variants have been expressed in *E. coli*. The identity of the expressed protein has been confirmed both by Western blotting and N-terminal sequencing. The protein is expressed in soluble form, but as a dimer which is somewhat surprising.

The HIS-tagged MUC-1 molecule has been purified by metal affinity chromatography. The amount of pure protein and the purity is currently unknown.

EXAMPLE 5

Breaking of autotolerance in a murine model system

CTL experiments where mice have been immunised with dendritic cells pulsed with both a class I and a class II epitope have previously shown an enhanced CTL induction when immunising as well as restimulating *in vitro* with both a class I and a class II peptide compared to an immunisation and re-stimulation with just a class I epitope. This situation is comparable with immunisation with an autovaccine, where a foreign class II epitope is inserted in a self protein. Uptake and processing

of these molecules by professional antigen presenting cells such as dendritic cells, leads to presentation of the foreign class II epitope together with some self class I epitopes. It is known that it is possible to elicit autoreactive CTL's, but 5 the presence of a foreign class II helper epitope very likely should enhance this CTL induction.

The potential advantage of the present invention for induction of self reactive CTLs is currently being investigated in ovalbumin transgenic mice. There exist four different trans-10 genic lines with different ovalbumin expression levels and tolerance states, cf. Kurts C et al. 1997, J. Exp. Med. **186**: 239-245 disclosing the RIP-mOVA transgenic mouse (expressing ovalbumin in pancreas, kidney and thymus and having a high degree of tolerance) and Kurts C et al., 1998, J. Exp. Med. 15 **188**: 409-414 disclosing the RIP-OVA^{low} and RIP-OVA^{high} transgenic mice, having low and high expression of ovalbumin, respectively. The last line, RIP-OVA^{int} which expresses ovalbumin at an intermediary level has been obtained from Dr. William R. Heath, co-author of the two above-mentioned references.

20 In the body there are different degrees of tolerance to different antigens. One of the least degrees of tolerance is found on circulating antigens in large amounts. These antigens will all enter thymus, where self reactive T-cells are deleted. These antigens are under "central tolerance". Tissue 25 specific antigens, on the other hand, do not directly enter the thymus and is generally under "peripheral tolerance", exerted by e.g. T-cell anergy.

Two ovalbumin AutoVac constructs have produced. They both relate to the sequence with accession No: J00845 in EMBL where 30 the sequence from P30 (SEQ ID NO: 14) have been inserted in two different positions.

In construct "OVA 3.1", P30 is inserted in the position that correspond to amino acid nos. 272-292 in ovalbumin. In construct "OVA 3.2", P30 is inserted in the position that corresponds to amino acid nos. 321-341 in ovalbumin. These constructs have been inserted in the vector pVax1 and used for DNA immunisation.

Mice have been immunised intradermally once with 100 ug each of DNA. Three weeks after this immunisation, the spleens were removed and a CTL assay was set up using target cells expressing the dominant ovalbumin epitope SIINFEKL and the scrambled FILKSINE peptide as control. The immunizations provided a clear CTL induction in wild-type C57BL/6 mice - as expected, since both ovalbumin and P30 are foreign in these mice.

We now intend to immunise the 4 lines of ovalbumin transgenic mice with these AutoVac constructs. The RIP-OVA^{low}, RIP-OVA^{int}, and RIP-OVA^{high} express increasing amounts of ovalbumin and have different degrees of tolerance and, as mentioned above, also RIP-mOVA has a high degree of tolerance.

In these 4 lines of transgenic mice, only P30 will be foreign. Ovalbumin is a self-antigen in these mice and this situation will therefore constitute a true autovaccination for CTL induction towards ovalbumin.

Preliminary results obtained in RIP-OVA^{low} mice having the lowest degree of "peripheral tolerance" to ovalbumin demonstrated that both the ovalbumin with inserted P30 and the naturally occurring ovalbumin molecules were capable of inducing CTL responses - it is expected that transgenic mice having higher degrees of tolerance will only be capable of mounting a CTL response against the modified ovalbumin molecules and not the naturally occurring form.

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CLAIMS

1. A method for inducing an immune response against a polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, the method comprising effecting simultaneous presentation by antigen presenting cells (APCs) of the animal's immune system of an immunogenically effective amount of
 - 1) at least one CTL epitope derived from the polypeptide antigen and/or at least one B-cell epitope derived from the cell-associated polypeptide antigen, and
 - 2) at least one first T helper cell epitope (T_H epitope) which is foreign to the animal.
2. A method for down-regulating a cell-associated polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, by inducing a specific cytotoxic T-lymphocyte (CTL) response against cells carrying the cell-associated polypeptide antigen on their surface or harbouring the cell-associated polypeptide antigen in their intracellular compartment, the method comprising effecting, in the animal, simultaneous presentation by a suitable antigen presenting cell (APC) of
 - 1) at least one CTL epitope derived from the cell-associated polypeptide antigen, and
 - 2) at least one first T-helper lymphocyte (T_H) epitope which is foreign to the animal.
3. The method according to claim 1 or 2, wherein said at least one CTL epitope when presented is associated with an MHC Class I molecule on the surface of the APC and/or wherein said at least one first foreign T_H epitope when presented is associated with an MHC Class II molecule on the surface of the APC.

4. The method according to any one of the preceding claims, wherein the APC is a dendritic cell or a macrophage.

5. The method according to any one of the preceding claims, wherein the polypeptide antigen is selected from a tumour-associated polypeptide antigen, a self-protein, a viral polypeptide antigen, and a polypeptide antigen derived from an intracellular parasite or bacterium.

6. The method according to any one of the preceding claims, wherein presentation by the APC of the CTL epitope and the first foreign T_H epitope is effected by presenting the animal's immune system with at least one first analogue of the polypeptide antigen, said first analogue comprising a variation of the amino acid sequence of the polypeptide antigen, said variation containing at least the CTL epitope and the first foreign T_H epitope.

7. The method according to claim 6, wherein the at least first analogue contains a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen.

20 8. The method according to claim 7, wherein the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 90% of the MHC-I haplotypes recognizing all known and predicted CTL epitopes in the cell-associated polypeptide antigen.

25 9. The method according to any one of claims 6-8, wherein substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue and/or wherein substantially all predicted CTL epitopes of the cell-associated polypeptide antigen are present in the at least first 30 analogue.

10. The method according to any one of claims 6-9, wherein the at least one first analogue further comprises a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that
5 immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-associated polypeptide antigen.

11. The method according to any one of the preceding claims, which comprises effecting presentation to the animal's immune system of an immunogenically effective amount of at least one second analogue of the polypeptide antigen, said second analogue containing a modification of the structure of the polypeptide antigen, said modification having as a result that immunization of the animal with the second analogue induces
15 production of antibodies against the cell-associated polypeptide antigen.

12. The method according to claim 11, wherein the modification comprises that at least one second foreign T_H epitope is included in the second analogue.

20 13. The method according to any one of claims 6-12, wherein the first and/or second analogue(s) comprise(s) a substantial fraction of the cell-associated polypeptide antigen's B-cell epitopes.

14. The method according to any one of claims 6-13, wherein
25 the variation and/or modification involves amino acid substitution and/or deletion and/or insertion and/or addition.

15. The method according to any one of claims 6-14, wherein the variation and/or modification comprises that

- at least one first moiety is included in the first and/or second analogue(s), said first moiety effecting targeting of the analogue to an antigen presenting cell (APC), and/or
- at least one second moiety is included in the first and/or

5 second analogue(s), said second moiety stimulating the immune system, and/or

- at least one third moiety is included in the first and/or second analogue(s), said third moiety optimizing presentation of the analogue to the immune system.

10 16. The method according to any one of claims 6-15, wherein the variation and/or modification includes duplication of at least one B-cell epitope or of at least one CTL epitope of the cell-associated polypeptide antigen

15 17. The method according to any one of claims 6-16, wherein the variation and/or modification includes introduction of a hapten.

18. The method according to any one of the preceding claims, wherein the first and/or second foreign T_H epitope(s) is/are immunodominant.

20 19. The method according to any one of the preceding claims, wherein the first and/or second foreign T_H epitope(s) is/are promiscuous.

25 20. The method according to any one of claims 12-19, wherein the first and/or second foreign T_H epitope(s) is/are selected from a natural T_H epitope and an artificial MHC-II binding peptide sequence.

21. The method according to claim 20, wherein the natural T_H epitope is selected from a Tetanus toxoid epitope such as P2

or P30, a diphtheria toxoid epitope, an influenza virus hemagglutinin epitope, and a *P. falciparum* CS epitope.

22. The method according to any one of claims 12-21, wherein the first and/or second T_H epitopes and/or first and/or second 5 and/or third moieties are present in the form of

- side groups attached covalently or non-covalently to suitable chemical groups in the amino acid sequence of the cell-associated polypeptide antigen or a subsequence thereof, and/or
- 10 - fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen.

23. The method according to claim 22, wherein the first moiety is a substantially specific binding partner for an APC specific surface antigen such as a carbohydrate for which there 15 is a receptor on the APC, e.g. mannan or mannose.

24. The method according to any one of claims 15-23, wherein the second moiety is a cytokine selected from interferon γ (IFN-γ), Flt3L, interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 20 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF), or an effective part thereof; a heat-shock protein selected from HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT), or an effective part thereof; or a hormone.

25 25. The method according to any one of claims 15-24, wherein the third moiety is a lipid such as a palmitoyl group, a myristyl group, a farnesyl group, a geranyl-geranyl group, a GPI-anchor, and an N-acyl diglyceride group.

26. The method according to claim any one of claims 6-25, 30 wherein the first and/or second analogue(s) has/have substan-

tially the overall tertiary structure of the cell-associated polypeptide antigen.

27. The method according to any one of claims 6-26, wherein presentation by the APC is effected by administering, to the 5 animal, an immunogenically effective amount of the at least one first analogue.

28. The method according to claim 27, wherein is also administered an immunologically effective amount of the at least one second analogue.

10 29. The method according to claim 27 or 28, wherein said at least one first and/or second analogue(s) is/are formulated together with a pharmaceutically and immunologically acceptable carrier and/or vehicle and, optionally an adjuvant.

15 30. The method according to claim 29, wherein said adjuvant facilitates uptake by APCs, such as dendritic cells, of the at least first and/or second analogues.

20 31. The method according to claim 30, wherein the adjuvant is selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ -inulin; and an encapsulating adjuvant.

25 32. The method according to claim 31, wherein the cytokine is as defined as in claim 24, or an effective part thereof, wherein the toxin is selected from the group consisting of listeriolytic (LLO), Lipid A (MPL, L180.5/RalLPS), and heat-labile enterotoxin, wherein the mycobacterial derivative is

selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE, wherein the immune targeting adjuvant is selected from the group consisting of CD40 ligand, CD40 anti-
5 bodies or specifically binding fragments thereof, mannose, a Fab fragment, and CTLA-4, wherein the oil formulation comprises squalene or incomplete Freund's adjuvant, wherein the polymer is selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a
10 plastic polymer such as; and latex such as latex beads, wherein the saponin is *Quillaja saponaria* saponin, Quil A, and QS21, and wherein the particle comprises latex or dextran.

33. The method according to any one of claims 27-32, which includes administration via a route selected from the oral
15 route and the parenteral route such as the intradermal, the subdermal, the intracutaneous, the subcutaneous; the peritoneal, the buccal, the sublingual, the epidural, the spinal, the anal, and the intracranial routes.

34. The method according to any of claim 27-33, which includes
20 at least one administration a year, such as at least 2, 3, 4, 5, 6, and 12 administrations a year.

35. The method according to any one of claims 1-5, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying a
25 nucleic acid fragment encoding and expressing the at least one CTL epitope and the at least one T_h epitope.

36. The method according to any one of claims 6-14, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at
30 least one nucleic acid fragment which encodes and expresses the at least first analogue.

37. The method according to any one of claims claims 15-26, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated

5 polypeptide antigen, and wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment encoding and expressing the first and/or second analogue.

38. The method according to any one of claims 11-14 or 36, 10 wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment which encodes and expresses the at least second analogue.

39. The method according to claim 38, wherein the non-patho- 15 genic microorganism or virus is administered once to the animal.

40. The method according to any one of claims 1-5, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment which encodes and expresses 20 the at least one CTL epitope and/or the at least one B-cell epitope, and the at least one first foreign T_H epitope.

41. The method according to any one of claims 6-14, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment encoding and expressing the 25 first analogue.

42. The method according to any one of claims claims 15-26, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated

polypeptide antigen, and wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment encoding and expressing the first and/or second analogue.

5 43. The method according to any one of claims 11-14 and 41, which further comprises *in vivo* introduction, into the APC, of at least one nucleic acid fragment encoding and expressing the second analogue.

10 44. The method according to any one of claims 1-5, wherein presentation is effected by *in vivo* co-introducing, into the APC, at least two nucleic acid fragments, wherein one encodes and expresses the at least one CTL epitope and wherein another encodes and expresses the at least one first foreign T_H epi-
15 tope, and wherein the first foreign T_H epitope is as defined in any one of claims 1, 2 and 21-24.

45. The method according to any one of claims 40-44, wherein the nucleic acid fragment(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids,
20 DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with a targeting carbohydrate, DNA formulated with Calcium precipitating agents, DNA coupled to
25 an inert carrier molecule, and DNA formulated with an adjuvant.

46. The method according to claim 45, wherein the adjuvant is selected from the group consisting of the adjuvants defined in any one of claims 30-32.

30 47. The method according to any one of claims 40-46, wherein the mode of administration is as defined in claim 33 or 34.

48. A method for selection of an immunogenic analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said immunogenic analogue being capable of inducing a CTL response in the animal
5 against cells displaying an MHC Class I molecule bound to an epitope derived from the cell-associated polypeptide antigen, the method comprising

- a) identifying at least one subsequence of the amino acid sequence of the cell-associated polypeptide antigen which does not contain known or predicted CTL epitopes,
- b) preparing at least one putatively immunogenic analogue of the cell-associated polypeptide antigen by introducing, in the amino acid sequence of the cell-associated polypeptide antigen, at least one T_H epitope foreign to the animal in a
15 position within the at least one subsequence identified in step a), and
- c) selecting the/those analogues prepared in step b) which are verifiably capable of inducing a CTL response in the animal.

20 49. The method according to claim 48, wherein

- 1) the subsequence identified in step a) further does not contain cysteine residues, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the pattern of cystein residues, and/or
- 25 2) the subsequence identified in step a) further does not contain known or predicted glycosylation sites, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the glycosylation pattern, and/or
- 3) the subsequence identified in step a) contributes significantly to a patophysiological effect exerted by the cell-associated polypeptide antigen, and wherein the introduc-
30

tion in step b) of the foreign T_H epitope reduces or abolishes said patophysiological effect, and/or

- 4) the subsequence identified in step a) is homologous to an amino acid sequence of a different protein antigen of the animal, and wherein the introduction of the T_H epitope in step b) substantially removes the homology, and/or
- 5) introduction in step b) of the foreign T_H epitope results in preservation of a substantial fraction of B-cell epitopes of the cell-associated polypeptide antigen.

10 50. The method according to claim 49, variant 5, wherein the analogue has the overall tertiary structure of the cell-associated polypeptide antigen.

51. A method for the preparation of cell producing an analogue of a cell-associated polypeptide antigen, the method comprising introducing, into a vector, a nucleic acid sequence encoding an analogue which has been selected according to the method of any one of claims 48-50 and transforming a suitable host cell with the vector.

20 52. A method for the preparation of an analogue of a cell-associated polypeptide antigen, the method comprising culturing the cell obtained according to the method of claim 51 under conditions facilitating expression of the nucleic acid sequence encoding the cell-associated polypeptide antigen, and recovering the analogue from the culture supernatant or from

25 the cells.

53. The method according to claim 52 which further comprises the step of purifying the recovered analogue and, optionally subjecting the purified product to artificial post-transla-

tional modifications such as refolding, treatment with enzymes, chemical modification, and conjugation.

54. The method according to any one of the preceding claims, wherein the weak cell-associated antigen is selected from the
5 group consisting of 5 alpha reductase, α -fetoprotein, AM-1 , APC, APRIL, BAGE, β -catenin, Bcl2, bcr-abl (b3a2), CA-125, CASP-8 / FLICE, Cathepsins, CD19, CD20, CD21, CD23, CD22, CD33, CD35, CD44, CD45, CD46; CD5, CD52, CD55 (791Tgp72), CD59, CDC27, CDK4, CEA, c-myc, Cox-2, DCC, DcR3, E6 / E7, EGFR, EMBP, Ena78, farsyl transferase, FGF8a or FGF8b, FLK-1/KDR, Folic Acid Receptor, G250, GAGE-Family, gastrin 17, Gastrin-releasing hormone (Bombesin), GD2 / GD3 / GM2, GnRH, GnTV, GP1, gp100 / Pmel 17, gp-100-in4, gp15, gp75 / TRP-1, hCG, Heparanase, Her2 / neu, HMTV, Hsp70, hTERT (telomerase),
10 IGFR1, IL-13R, iNOS, Ki 67, KIAA0205, K-ras, H-ras, N-ras, KSA (CO17-1A), LDLR-FUT, MAGE Family (MAGE-1, MAGE-2, MAGE-3, etc), Mammaglobin, MAP17, Melan-A / MART-1, mesothelin, MIC A/B, MT-MMP's, Mox1, Mucin such as MUC-1, MUC-2, MUC-3, and MUC-4 being abberantly glycosylated, MUM-1, NY-ESO-1,
15 Osteonectin, p15, P170 / MDR1, p53, p97 / melanotransferrin, PAI-1, PDGF, Plasminogen (uPA), PRAME, Probasin, Progenipoitin, PSA, PSM, RAGE-1, Rb, RCAS1, SART-1, SSX gene family, STAT3, STn (mucin assoc.), TAG-72, TGF- α , TGF- β , Thymosin β 15, TNF- α , TPA, TPI, TRP-2, Tyrosinase, VEGF, ZAG, p16INK4,
20 and Glutathione S-transferase.
25

55. The method according to claim 54, wherein the cell-associated polypeptide antigen is human PSM.

56. The method according to claim 55, wherein the foreign T-cell epitope is introduced in a part of the PSM amino acid
30 sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699.

57. The method according to claim 55 or 56 used in the treatment or amelioration of prostate cancer.

58. The method according to claim 54, wherein the cell-associated polypeptide antigen is fibroblast growth factor 8b (FGF8b).

59. The method according to claim 58, where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215
10 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177, and wherein the introduction preferably does not substantially involve amino acids 26-45 and amino acids 186-215.

15 60. The method according to claim 58 or 59 used in the treatment or amelioration of cancer such as prostate cancer and breast cancer.

61. The method according to claim 54, wherein the cell-associated polypeptide antigen is Her2.

20 62. The method according to claim 61, wherein the foreign T-cell epitope is introduced in a part of the Her2 amino acid sequence defined by SEQ ID NO: 3 positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-593
25 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or 710-730.

72-86 and/or 146-160 and/or 221-235 and/or 257-271 and/or 387-401.

63. The method according to claim 61 or 62 used in the treatment or amelioration of breast cancer.

64. An analogue of human PSM which is immunogenic in humans,
5 said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of PSM and including at least one foreign T_H epitope as defined in any one of claims 18-21.

65. The analogue according to claim 64, wherein the at least 10 one foreign T_H epitope is present as an insertion in the PSM amino acid sequence or as a substitution of part of the PSM amino acid sequence or as the result of deletion of part of the PSM amino acid sequence.

66. The analogue according to claim 65, wherein the foreign T_H 15 epitope is introduced in the positions defined in claim 56.

67. An analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign T_H epitope as defined in any one of claims 20 18-21.

68. The analogue according to claim 67, wherein the at least one foreign T_H epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of 25 the Her2 amino acid sequence.

69. The analogue according to claim 68, wherein the foreign T_H epitope is introduced in the positions defined in claim 62.

70. An analogue of human/murine FGF8b which is immunogenic in humans, said analogue comprising a substantial part of all

known and predicted CTL and B-cell epitopes of FGF8b and including at least one foreign T_H epitope as defined in any one of claims 18-21.

71. The analogue according to claim 70, wherein the at least 5 one foreign T_H epitope is present as an insertion in the FGF8b amino acid sequence or as a substitution of part of the FGF8b amino acid sequence or as the result of deletion of part of the FGF8b amino acid sequence.

72. The analogue according to claim 71, wherein the foreign T_H 10 epitope is introduced in the positions defined in claim 59.

73. An immunogenic composition which comprises, as an effective immunogenic agent the analogue according to any one of claims 64-72 in admixture with a pharmaceutically and immuno- logically acceptable carrier or vehicle, and optionally an 15 adjuvant.

74. A nucleic acid fragment which encodes an analogue according to any one of claims 64-72.

75. A vector carrying the nucleic acid fragment according to claim 74.

20 76. The vector according to claim 75 which is capable of autonomous replication.

77. The vector according to claim 75 or 76 which is selected from the group consisting of a plasmid, a phage, a cosmid, a mini-chromosome, and a virus.

25 78. The vector according to any one of claims 75-77, comprising, in the 5'-3' direction and in operable linkage, a promoter for driving expression of the nucleic acid fragment

according to claim 74, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment according to claim 74, and optionally a nucleic acid sequence encoding a terminator.

79. The vector according to any one of claims 75-78 which, when introduced into a host cell, is integrated in the host cell genome or is not capable of being integrated in the host cell genome.

10 80. A transformed cell carrying the vector of any one of claims 75-79.

81. A composition for inducing production of antibodies against PSM, Her2 or FGF8b, the composition comprising
1) a nucleic acid fragment according to claim 74 or a vector
15 according to any one of claims 75-79, and
2) a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or adjuvant.

82. A stable cell line which carries the vector according to any one of claims 75-79 and which expresses the nucleic acid
20 fragment according to claim 74, and which optionally secretes or carries the analogue according to any one of claims 64-72 on its surface.

83. A method for the preparation of the cell according to claim 80, the method comprising transforming a host cell with
25 the nucleic acid fragment according to claim 74 or with the vector according to any one of claims 75-79.

1/6

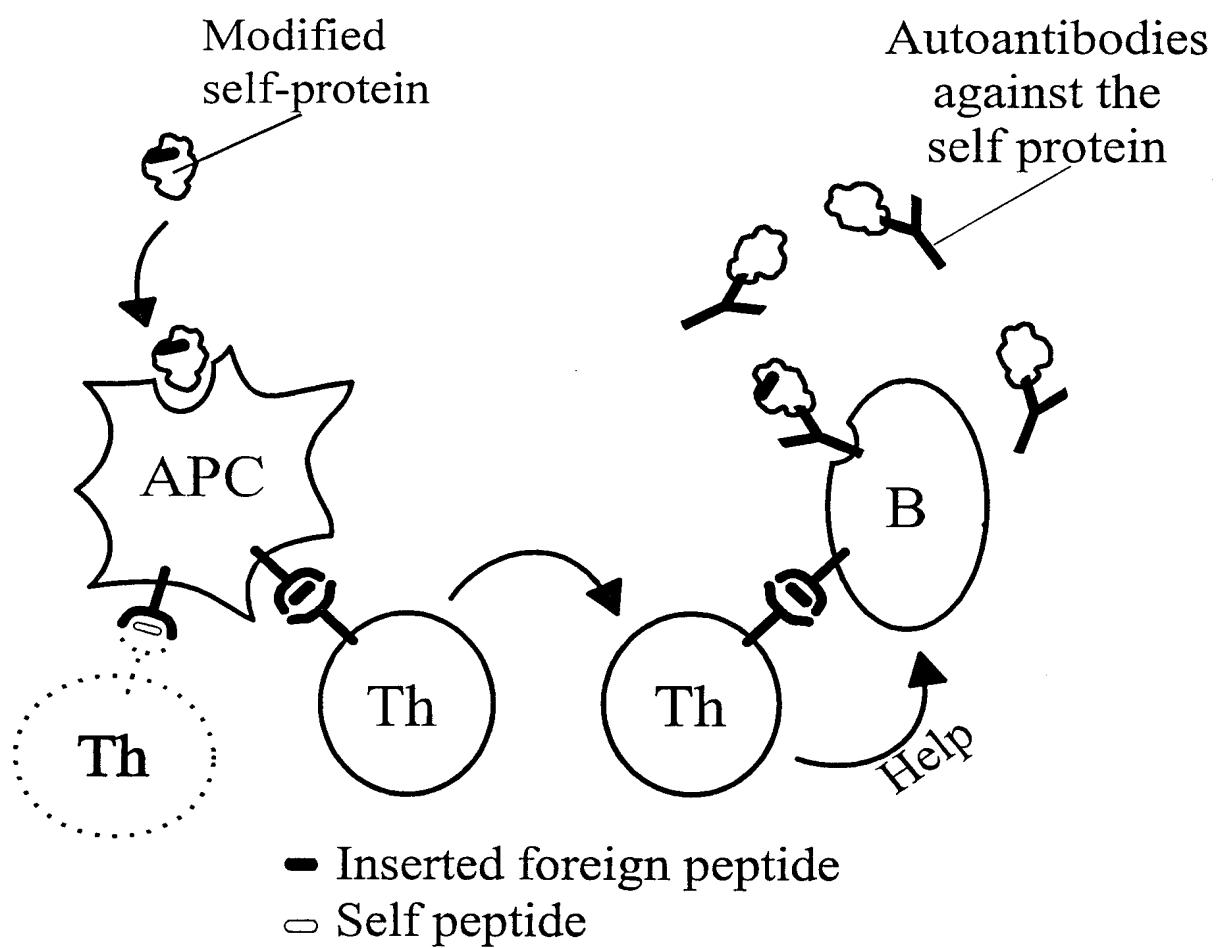
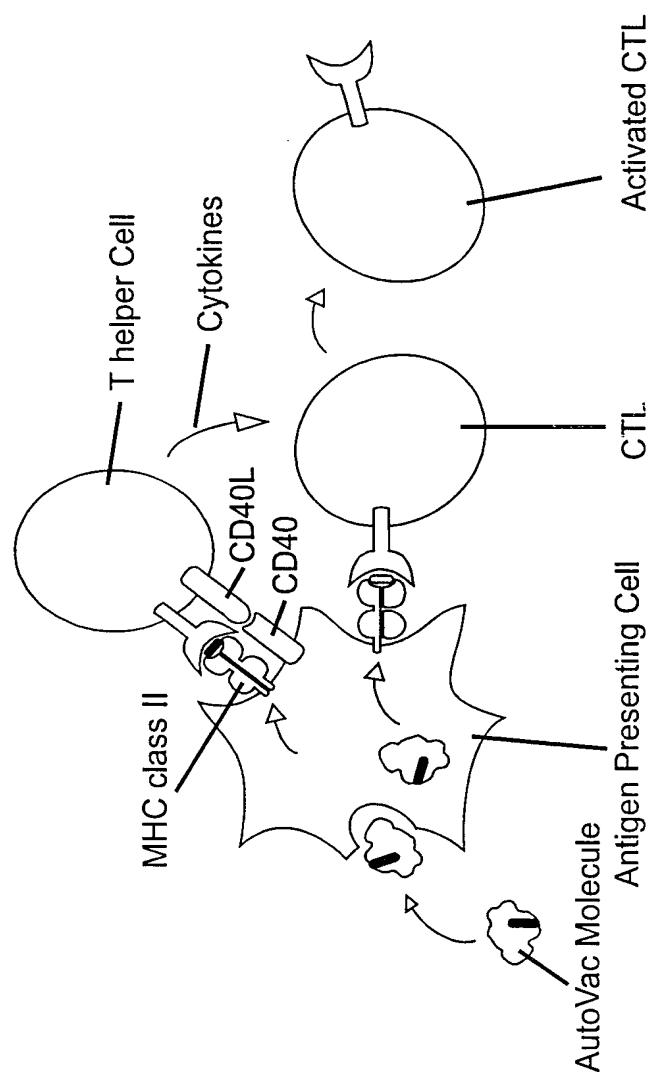
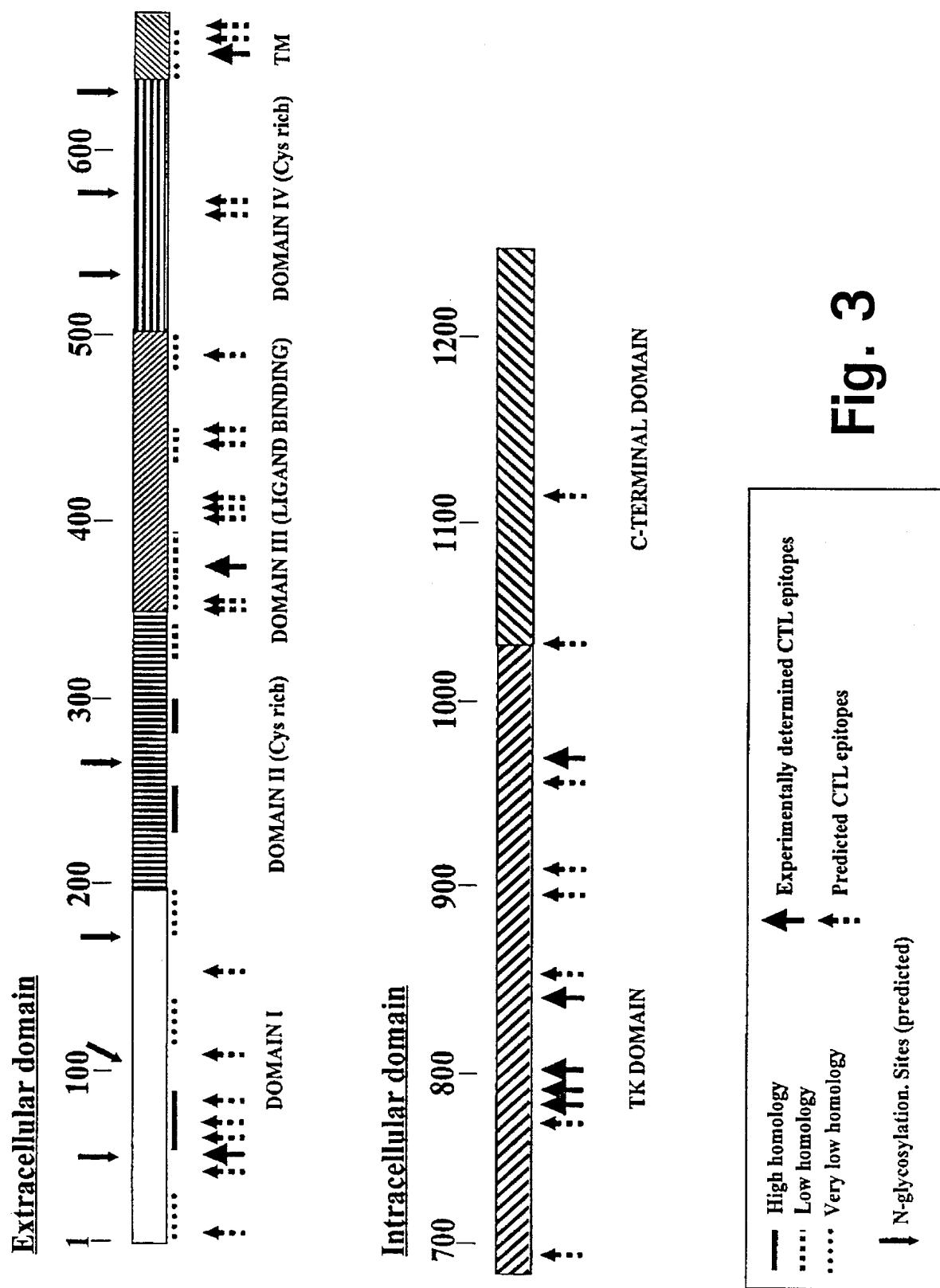


Fig. 1

2/6

**Fig. 2**

3/6

**Fig. 3**

4/6

**Human PSM constructs
(schematic representation)**

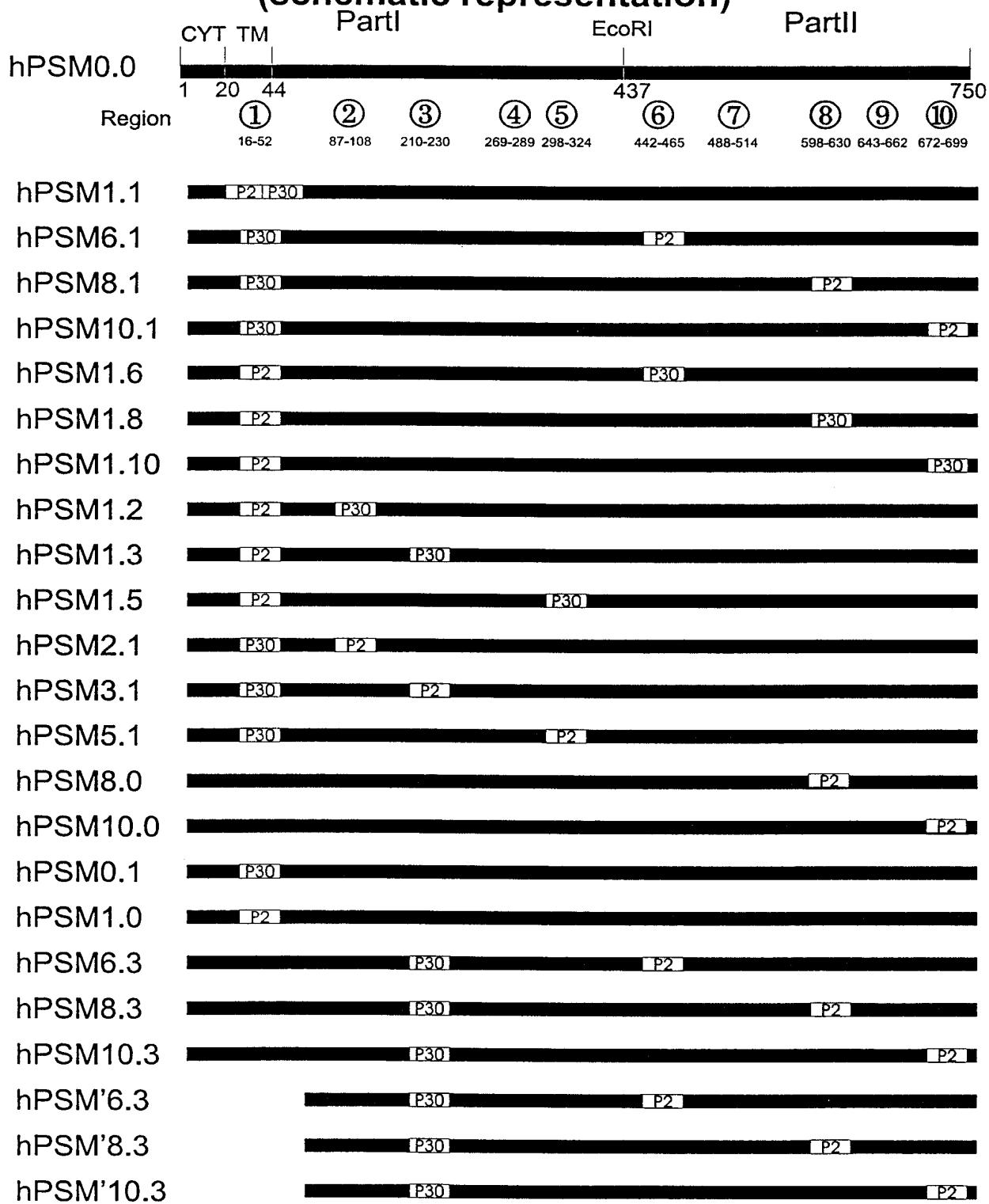


Fig. 4

5/6

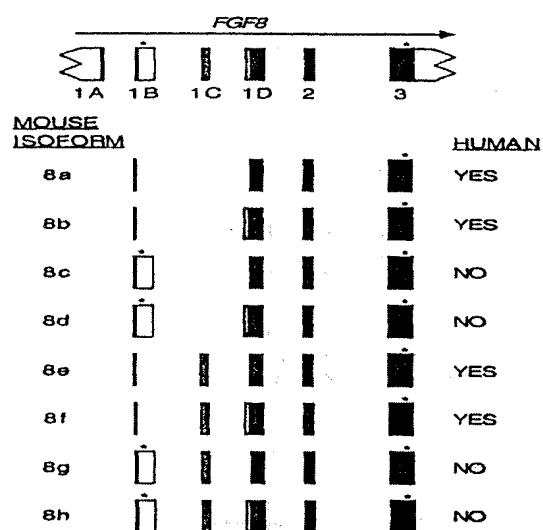
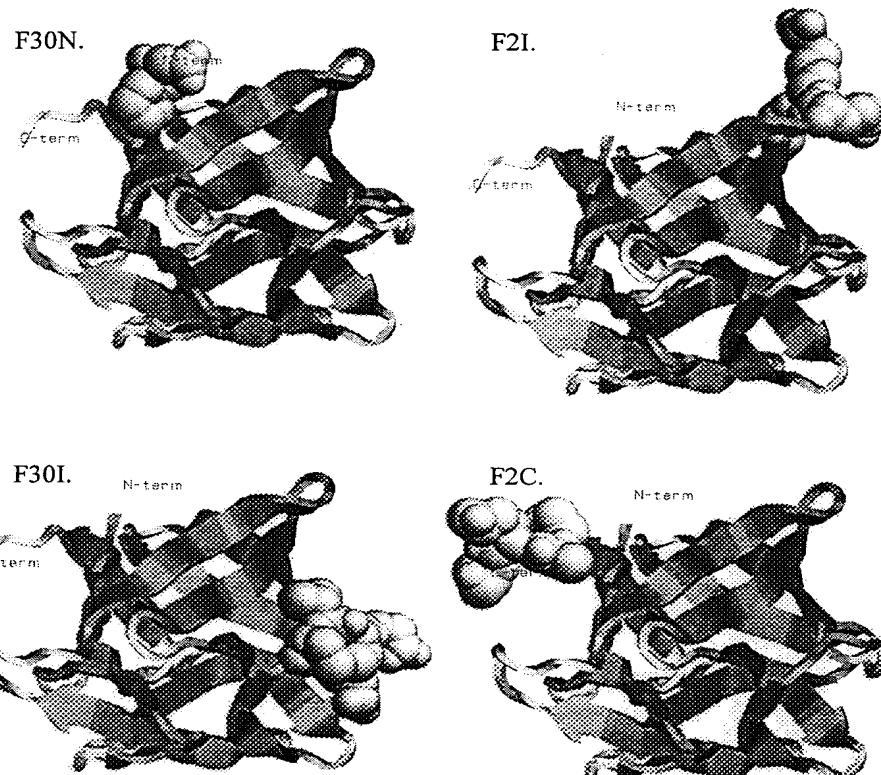


Fig. 5A

FGF8e and -f	FGF8b and -f
MGSPRSALSC LLLHLLVLCL QAQEGPGRGP ALGRELASF RAGREPQGVS	QQVTVQSSPN 31
FTQHVREQSL VTDQLSRRLI RTYQLYSRTS GKHVQVLANK RINAMAEDGD	PFALKLIVETD 91
TFGSRVRVRG AETGLYICMN KKGKLIAKSN GKGKD_CVFTE IVLENÑYTAL	QNAKYEGWYM 151
AFTRKGRPRK GSKTRQHQRE VHFMKRLPRG HHTTEQSLRF EFLNYPPFTR SLRGSQRTWA	211
PEPR	215

Fig. 5B

6/6



WT	<u>MGS</u> PRSA <u>L</u> SCL <u>LL</u> H <u>LL</u> V <u>L</u> C <u>I</u> Q <u>A</u> Q <u>V</u> T <u>V</u> Q <u>S</u> S <u>P</u> N <u>F</u> T <u>Q</u> H <u>V</u> R <u>E</u> Q <u>S</u> L <u>V</u> T <u>D</u> Q <u>L</u> S <u>R</u> R <u>L</u> I <u>R</u> T <u>Y</u> Q <u>L</u> Y <u>S</u> R <u>T</u> S <u>G</u> K <u>H</u> Q	66
F30N	MAQ <u>V</u> T <u>V</u> <u>FNN</u> F <u>NFTV</u> S <u>FWL</u> R <u>VPK</u> V <u>SASH</u> <u>LERR</u> I <u>R</u> T <u>Y</u> Q <u>L</u> Y <u>S</u> R <u>T</u> S <u>G</u> K <u>H</u> Q	46
F2I	MAQ <u>V</u> T <u>V</u> Q <u>S</u> S <u>P</u> N <u>F</u> T <u>Q</u> H <u>V</u> R <u>E</u> Q <u>S</u> L <u>V</u> T <u>D</u> Q <u>L</u> S <u>R</u> R <u>L</u> I <u>R</u> T <u>Y</u> Q <u>L</u> Y <u>S</u> R <u>T</u> S <u>G</u> K <u>H</u> Q	46
F30I	MAQ <u>V</u> T <u>V</u> Q <u>S</u> S <u>P</u> N <u>F</u> T <u>Q</u> H <u>V</u> R <u>E</u> Q <u>S</u> L <u>V</u> T <u>D</u> Q <u>L</u> S <u>R</u> R <u>L</u> I <u>R</u> T <u>Y</u> Q <u>L</u> Y <u>S</u> R <u>T</u> S <u>G</u> K <u>H</u> Q	46
F2C	MAQ <u>V</u> T <u>V</u> Q <u>S</u> S <u>P</u> N <u>F</u> T <u>Q</u> H <u>V</u> R <u>E</u> Q <u>S</u> L <u>V</u> T <u>D</u> Q <u>L</u> S <u>R</u> R <u>L</u> I <u>R</u> T <u>Y</u> Q <u>L</u> Y <u>S</u> R <u>T</u> S <u>G</u> K <u>H</u> Q	46
WT	VLANKRINAMAEDGDPFAKLIVETDTF	GSRVVRVRAETGLYICMNNKKGKLIAK 119
F30N	VLANKRINAMAEDGDPFAKLIVETDTF	GSRVVRVRAETGLYICMNNKKGKLIAK 99
F2I	VLANKRINAMAEDGDPFAKLIVET <u>D<u>OYIKANSKFIGITEL</u></u> GSRVVRVRAETGLYICMNNKKGKLIAK 112	112
F30I	VLANKRINAMAEDGDPFAKLIVETDTF	GSRVVRVRAETGLYICMNNKKGKLIAK 99
F2C	VLANKRINAMAEDGDPFAKLIVETDTF	GSRVVRVRAETGLYICMNNKKGKLIAK 99
WT	SNG	KGKDCVFTIEGLENNNTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 167
F30N	SNG	KGKDCVFTIEGLENNNTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147
F2I	SNG	KGKDCVFTIEGLENNNTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 160
F30I	<u>SNG</u> <u>FNN</u> F <u>NFTV</u> S <u>FWL</u> R <u>VPK</u> V <u>SASH</u> <u>LED</u> CVFTIEGLENNNTALQNAKYEGWYMAFTRKGRPRKGSKTRQ	165
F2C	SNG	KGKDCVFTIEGLENNNTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147
WT	H <u>Q</u> RE <u>V</u> H <u>F</u> M <u>K</u> R <u>L</u> P <u>R</u> G <u>H</u> HTTE <u>Q</u> SL <u>R</u> FE <u>F</u> LN <u>Y</u> PP <u>F</u> T	R <u>S</u> L <u>R</u> G <u>S</u> Q <u>R</u> T <u>W</u> A PEPR 215
F30N	H <u>Q</u> RE <u>V</u> H <u>F</u> M <u>K</u> R <u>L</u> P <u>R</u> G <u>H</u> HTTE <u>Q</u> SL <u>R</u> FE <u>F</u> LN <u>Y</u> PP <u>F</u> T	R <u>S</u> L <u>R</u> G <u>S</u> Q <u>R</u> T <u>W</u> A PEPR 195
F2I	H <u>Q</u> RE <u>V</u> H <u>F</u> M <u>K</u> R <u>L</u> P <u>R</u> G <u>H</u> HTTE <u>Q</u> SL <u>R</u> FE <u>F</u> LN <u>Y</u> PP <u>F</u> T	R <u>S</u> L <u>R</u> G <u>S</u> Q <u>R</u> T <u>W</u> A PEPR 208
F30I	H <u>Q</u> RE <u>V</u> H <u>F</u> M <u>K</u> R <u>L</u> P <u>R</u> G <u>H</u> HTTE <u>Q</u> SL <u>R</u> FE <u>F</u> LN <u>Y</u> PP <u>F</u> T	R <u>S</u> L <u>R</u> G <u>S</u> Q <u>R</u> T <u>W</u> A PEPR 213
F2C	H <u>Q</u> RE <u>V</u> H <u>F</u> M <u>K</u> R <u>L</u> P <u>R</u> G <u>H</u> HTTE <u>Q</u> SL <u>R</u> FE <u>F</u> LN <u>Y</u> PP <u>F</u> <u>TOYIKANSKFIGITEL</u> PEPR	199

Fig. 6

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<110> M&E Biotech A/S
STEINAA, Lucilla
NIELSEN, Klaus Gregorius
DALUM, Iben
HAANING, Jesper
LEACH, Dana
BIRK, Peter
MOURITSEN, Soren
GAUTAM, Anand
KARLSSON, Gunilla

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1 5 10 15

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Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe
20 25 30

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Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe Ile Lys Ser Ser Asn Glu			
35	40	45	
gct act aac att act cca aag cat aat atg aaa gca ttt ttg gat gaa		192	
Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu			
50	55	60	
ttg aaa gct gag aac atc aag aag ttc tta tat aat ttt aca cag ata		240	
Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile			
65	70	75	80
cca cat tta gca gga aca gaa caa aac ttt cag ctt gca aag caa att		288	
Pro His Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile			
85	90	95	
caa tcc cag tgg aaa gaa ttt ggc ctg gat tct gtt gag cta gca cat		336	
Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His			
100	105	110	
tat gat gtc ctg ttg tcc tac cca aat aag act cat ccc aac tac atc		384	
Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile			
115	120	125	
tca ata att aat gaa gat gga aat gag att ttc aac aca tca tta ttt		432	
Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe			
130	135	140	
gaa cca cct cca gga tat gaa aat gtt tcg gat att gta cca cct		480	
Glu Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro			
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Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr			
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gtt aac tat gca cga act gaa gac ttc ttt aaa ttg gaa cgg gac atg		576	
Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met			
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Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val			
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Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr			
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Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly			
275	280	285	
ctt cca agt att cct gtt cat cca att gga tac tat gat gca cag aag		912	
Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys			
290	295	300	
ctc cta gaa aaa atg ggt ggc tca gca cca cca gat agc agc tgg aga		960	
Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg			
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Gly Ser Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn			
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Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val			
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Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro			
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Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly			
370	375	380	
ggt att gac cct cag agt gga gca gct gtt gtt cat gaa att gtg agg		1200	
Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg			
385	390	395	400
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Ser Phe Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile			
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Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr			
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Glu Trp Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala			
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Asp Cys Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu			
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Trp Thr Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile			
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Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn			
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Lys Phe Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu			
545	550	555	560
ttg gtg gaa aag ttt tat gat cca atg ttt aaa tat cac ctc act gtg			1728
Leu Val Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val			
565	570	575	
gcc cag gtt cga gga ggg atg gtg ttt gag cta gcc aat tcc ata gtg			1776
Ala Gln Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val			
580	585	590	
ctc cct ttt gat tgt cga gat tat gct gta gtt tta aga aag tat gct			1824
Leu Pro Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala			
595	600	605	
gac aaa atc tac agt att tct atg aaa cat cca cag gaa atg aag aca			1872
Asp Lys Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr			
610	615	620	
tac agt gta tca ttt gat tca ctt ttt tct gca gta aag aat ttt aca			1920
Tyr Ser Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr			
625	630	635	640
gaa att gct tcc aag ttc agt gag aga ctc cag gac ttt gac aaa agc			1968
Glu Ile Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser			
645	650	655	

aac cca ata gta tta aga atg atg aat gat caa ctc atg ttt ctg gaa			2016
Asn Pro Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu			
660	665	670	
aga gca ttt att gat cca tta ggg tta cca gac agg cct ttt tat agg			2064
Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg			
675	680	685	
cat gtc atc tat gct cca agc agc cac aac aag tat gca ggg gag tca			2112
His Val Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser			
690	695	700	
ttc cca gga att tat gat gct ctg ttt gat att gaa agc aaa gtg gac			2160
Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp			
705	710	715	720
cct tcc aag gcc tgg gga gaa gtg aag aga cag att tat gtt gca gcc			2208
Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala			
725	730	735	
ttc aca gtg cag gca gct gca gag act ttg agt gaa gta gcc taa			2253
Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala			
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Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe			
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Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe Ile Lys Ser Ser Asn Glu			
35	40	45	
Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu			
50	55	60	
Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile			
65	70	75	80
Pro His Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile			
85	90	95	
Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His			
100	105	110	

Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile
115 120 125

Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe
130 135 140

Glu Pro Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro
145 150 155 160

Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr
165 170 175

Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met
180 185 190

Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val
195 200 205

Phe Arg Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly
210 215 220

Val Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys
225 230 235 240

Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Val Gln Arg Gly
245 250 255

Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr
260 265 270

Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly
275 280 285

Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys
290 295 300

Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg
305 310 315 320

Gly Ser Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn
325 330 335

Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val
340 345 350

Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro
355 360 365

Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly
370 375 380

Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg
385 390 395 400

His	Val	Ile	Tyr	Ala	Pro	Ser	Ser	His	Asn	Lys	Tyr	Ala	Gly	Glu	Ser	
690						695						700				

Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp
705 710 715 720

Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala
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Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
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Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
-20           -15           -10

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ccc ccc gga gcc gcg agc acc caa gtg tgc acc ggc aca gac atg aag 96
Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
          -5           -1   1           5

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ctg cg10 ctc c15 ct20 g25 cc144
Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His

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ctc tac cag ggc tgc cag gtg gtg cag gga aac ctg gaa ctc acc tac 192
 Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
 30 35 40

ctg ccc acc aat gcc agc ctg tcc ttc ctg cag gat atc cag gag gtg 240
 Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
 45 50 55

cag ggc tac gtg ctc atc gct cac aac caa gtg agg cag gtc cca ctg 288
Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
60 65 70

cag agg ctg cg att gtg cga ggc acc cag ctc ttt gag gac aac tat 336
Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
75 80 85

gcc ctg gcc gtg cta gac aat gga gac ccg ctg aac aat acc acc cct			384
Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro			
90	95	100	105
gtc aca ggg gcc tcc cca gga ggc ctg cg ^g gag ctg cag ctt cga agc			432
Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser			
110	115	120	
ctc aca gag atc ttg aaa gga ggg gtc ttg atc cag cg ^g aac ccc cag			480
Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln			
125	130	135	
ctc tgc tac cag gac acg att ttg tgg aag gac atc ttc cac aag aac			528
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn			
140	145	150	
aac cag ctg gct ctc aca ctg ata gac acc aac cg ^c tct cg ^g gcc tgc			576
Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys			
155	160	165	
cac ccc tgt tct ccg atg tgt aag ggc tcc cg ^c tgc tgg gga gag agt			624
His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser			
170	175	180	185
tct gag gat tgt cag agc ctg acg cg ^c act gtc tgt gcc ggt ggc tgt			672
Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys			
190	195	200	
gcc cg ^c tgc aag ggg cca ctg ccc act gac tgc tgc cat gag cag tgt			720
Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys			
205	210	215	
gct gcc ggc tgc acg ggc ccc aag cac tct gac tgc ctg gcc tgc ctc			768
Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu			
220	225	230	
cac ttc aac cac agt gg ^c atc tgt gag ctg cac tgc cca gcc ctg gtc			816
His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val			
235	240	245	
acc tac aac aca gac acg ttt gag tcc atg ccc aat ccc gag ggc cg ^g			864
Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg			
250	255	260	265
tat aca ttc ggc gcc agc tgt gtg act gcc tgt ccc tac aac tac ctt			912
Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu			
270	275	280	
tct acg gac gtg gga tcc tgc acc ctc gtc tgc ccc ctg cac aac caa			960
Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln			
285	290	295	

gag gtg aca gca gag gat gga aca cag cgg tgt gag aag tgc agc aag		1008
Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys		
300	305	310
ccc tgt gcc cga gtg tgc tat ggt ctg ggc atg gag cac ttg cga gag		1056
Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu		
315	320	325
gtg agg gca gtt acc agt gcc aat atc cag gag ttt gct ggc tgc aag		1104
Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys		
330	335	340
aag atc ttt ggg agc ctg gca ttt ctg ccg gag agc ttt gat ggg gac		1152
Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp		
350	355	360
cca gcc tcc aac act gcc ccg ctc cag cca gag cag ctc caa gtg ttt		1200
Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe		
365	370	375
gag act ctg gaa gag atc aca ggt tac cta tac atc tca gca tgg ccg		1248
Glu Thr Leu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro		
380	385	390
gac agc ctg cct gac ctc agc gtc ttc cag aac ctg caa gta atc cgg		1296
Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg		
395	400	405
gga cga att ctg cac aat ggc gcc tac tcg ctg acc ctg caa ggg ctg		1344
Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu		
410	415	420
425		
ggc atc agc tgg ctg ggg ctg cgc tca ctg agg gaa ctg ggc agt gga		1392
Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly		
430	435	440
ctg gcc ctc atc cac cat aac acc cac ctc tgc ttc gtg cac acg gtg		1440
Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val		
445	450	455
ccc tgg gac cag ctc ttt cgg aac ccg cac caa gct ctg ctc cac act		1488
Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr		
460	465	470
gcc aac cgg cca gag gac gag tgt gtg ggc gag ggc ctg gcc tgc cac		1536
Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His		
475	480	485
cag ctg tgc gcc cga ggg cac tgc tgg ggt cca ggg ccc acc cag tgt		1584
Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys		
490	495	500
505		

gtc aac tgc agc cag ttc ctt cg ^g ggc cag gag tgc gtg gag gaa tgc		1632	
Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys			
510	515	520	
cga gta ctg cag ggg ctc ccc agg gag tat gtg aat gcc agg cac tgt		1680	
Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys			
525	530	535	
ttg ccg tgc cac cct gag tgt cag ccc cag aat ggc tca gtg acc tgt		1728	
Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys			
540	545	550	
ttt gga ccg gag gct gac cag tgt gtg gcc tgt gcc cac tat aag gac		1776	
Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp			
555	560	565	
cct ccc ttc tgc gtg gcc cgc tgc ccc agc ggt gtg aaa cct gac ctc		1824	
Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu			
570	575	580	585
tcc tac atg ccc atc tgg aag ttt cca gat gag gag ggc gca tgc cag		1872	
Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln			
590	595	600	
cct tgc ccc atc aac tgc acc cac tcc tgt gtg gac ctg gat gac aag		1920	
Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys			
605	610	615	
ggc tgc ccc gcc gag cag aga gcc agc cct ctg acg tcc atc gtc tct		1968	
Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser			
620	625	630	
g ^c g gt ^g gtt g ^c g att ctg ctg gtc gtg gtc ttg g ^g g gtg gtc tt ^t g ^g g		2016	
Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly			
635	640	645	
atc ctc atc aag cga cgg cag cag aag atc cgg aag tac acg atg cgg		2064	
Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg			
650	655	660	665
aga ctg ctg cag gaa acg gag ctg gtg gag c ^g g ctg aca cct agc gga		2112	
Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly			
670	675	680	
g ^c g atg ccc aac cag g ^c g cag atg cgg atc ctg aaa gag acg gag ctg		2160	
Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu			
685	690	695	
agg aag gtg aag gtg ctt gga tct ggc gct tt ^t ggc aca gtc tac aag		2208	
Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys			
700	705	710	

ggc atc tgg atc cct gat ggg gag aat gtg aaa att cca gtg gcc atc		2256
Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile		
715 720 725		
aaa gtg ttg agg gaa aac aca tcc ccc aaa gcc aac aaa gaa atc tta		2304
Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu		
730 735 740 745		
gac gaa gca tac gtg atg gct ggt gtg ggc tcc cca tat gtc tcc cgc		2352
Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg		
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Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu		
765 770 775		
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Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg		
780 785 790		
ctg ggc tcc cag gac ctg aac tgg tgt atg cag att gcc aag ggg		2496
Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly		
795 800 805		
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Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala		
810 815 820 825		
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Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe		
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Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp		
845 850 855		
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Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg		
860 865 870		
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Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val		
875 880 885		
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Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala		
890 895 900 905		
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Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro		
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 Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
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 Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
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ctg gag gac gat gac atg ggg gac ctg gtg gat gct gag gag tat ctg 3072
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 1100 1105 1110

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 Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
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Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu	
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agg gcc aag act ctc tcc cca ggg aag aat ggg gtc gtc aaa gac gtt	3552
Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val	
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ttt gcc ttt ggg ggt gcc gtg gag aac ccc gag tac ttg aca ccc cag	3600
Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln	
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Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala	
1180 1185 1190	
ttc gac aac ctc tat tac tgg gac cag gac cca cca gag cgg ggg gct	3696
Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala	
1195 1200 1205	
cca ccc agc acc ttc aaa ggg aca cct acg gca gag aac cca gag tac	3744
Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr	
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35 40 45	
Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr	
50 55 60	
Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val	
65 70 75 80	
Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu	
85 90 95	

15

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
100 105 110

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
115 120 125

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
130 135 140

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145 150 155 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
165 170 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
180 185 190

His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
195 200 205

Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
210 215 220

Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225 230 235 240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
245 250 255

His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
260 265 270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
275 280 285

Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
290 295 300

Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305 310 315 320

Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
325 330 335

Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
340 345 350

Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
355 360 365

Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
370 375 380

Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
 385 390 395 400

 Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
 405 410 415

 Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
 420 425 430

 Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
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 Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
 450 455 460

 Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
 465 470 475 480

 Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
 485 490 495

 Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
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 Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
 515 520 525

 Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
 530 535 540

 Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
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 Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
 565 570 575

 Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
 580 585 590

 Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
 595 600 605

 Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
 610 615 620

 Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
 625 630 635 640

 Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser
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 Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
 660 665 670

Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
675 680 685

Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
690 695 700

Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705 710 715 720

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
725 730 735

Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
740 745 750

Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
755 760 765

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
770 775 780

Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785 790 795 800

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
805 810 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
820 825 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
835 840 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
850 855 860

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865 870 875 880

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
885 890 895

Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
900 905 910

Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
915 920 925

Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
930 935 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945 950 955 960

Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
 965 970 975
 Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
 980 985 990
 Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
 995 1000 1005
 Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
 1010 1015 1020
 Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
 025 1030 1035 1040
 Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
 1045 1050 1055
 Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Ala Pro Arg
 1060 1065 1070
 Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
 1075 1080 1085
 Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
 1090 1095 1100
 Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
 1095 1110 1115 1120
 Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
 1125 1130 1135
 Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
 1140 1145 1150
 Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
 1155 1160 1165
 Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
 1170 1175 1180
 Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
 1185 1190 1195 1200
 Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
 1205 1210 1215
 Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
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 Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
 1235 1240 1245

Leu Gly Leu Asp Val Pro Val
1250 1255

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Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu
1 5 10 15

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gtt ctc tgc ctc caa gcc cag gta act gtt cag tcc tca cct aat ttt      96
Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
                           20                      25                      30

```

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aca cag cat gtg agg gag cag agc ctg gtg acg gat cag ctc agc cgc 144
Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
          35           40           45

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cgc ctc atc cg acc tac cag ctc tac agc cgc acc acc agc ggg aag cac    192
Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
      50          55          60

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gtg cag gtc ctg gcc aac aag cgc atc aac gcc atg gca gaa gac gga 240
Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
   65           70           75           80

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gac ccc ttc gcg aag ctc att gtg gag acc gat act ttt gga agc aga	288	
Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg		
85	90	95

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gtc cga gtt cgc ggc gca gag aca ggt ctc tac atc tgc atg aac aag      336
Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
          100          105          110

```

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aag ggg aag cta att gcc aag agc aac ggc aaa ggc aag gac tgc gta      384
Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
          115           120           125

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ttc aca gag atc gtg ctg gag aac aac tac acg gcg ctg cag aac gcc      432
Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
          130           135           140

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20

aag tac gag ggc tgg tac atg gcc ttt acc cgc aag ggc cg ^g ccc cgc	480
Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg	
145 150 155 160	
aag ggc tcc aag acg cgc cag cat cag cgc gag gtg cac ttc atg aag	528
Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys	
165 170 175	
cgc ctg ccg cgg ggc cac cac acc acc gag cag agc ctg cgc ttc gag	576
Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu	
180 185 190	
ttc ctc aac tac ccg ccc ttc acg cgc agc ctg cgc ggc agc cag agg	624
Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg	
195 200 205	
act tgg gcc ccg gag ccc cga tag	648
Thr Trp Ala Pro Glu Pro Arg	
210 215	

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<212> PRT

<213> Homo sapiens

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Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu	
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Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe	
20 25 30	

Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg	
35 40 45	

Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His	
50 55 60	

Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly	
65 70 75 80	

Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg	
85 90 95	

Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys	
100 105 110	

Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val	
115 120 125	

Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala	
130 135 140	

21

Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
 145 150 155 160

Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
 165 170 175

Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
 180 185 190

Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
 195 200 205

Thr Trp Ala Pro Glu Pro Arg
 210 215

<210> 7

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Met Trp Asn Ala Leu Gln Asp Arg Asp Ser Ala Glu Val Leu Gly His	
1 5 10 15	

cgc cag cgc tgg ctc cgt gtt ggg aca ctg gtg ctg gct tta acc gga	96
Arg Gln Arg Trp Leu Arg Val Gly Thr Leu Val Leu Ala Leu Thr Gly	
20 25 30	

acc ttc ctc att ggc ttc ctc ttt ggg tgg ttt ata aaa cct tcc aat	144
Thr Phe Leu Ile Gly Phe Leu Phe Gly Trp Phe Ile Lys Pro Ser Asn	
35 40 45	

gaa gct act ggt aat gtt tcc cat tct ggc atg aag aag gag ttt ttg	192
Glu Ala Thr Gly Asn Val Ser His Ser Gly Met Lys Lys Glu Phe Leu	
50 55 60	

cat gaa ttg aag gct gag aac atc aaa aaa ttt tta tac aat ttc aca	240
His Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr	
65 70 75 80	

cgg aca cca cac ttg gca gga aca caa aat aat ttt gag ctt gca aag	288
Arg Thr Pro His Leu Ala Gly Thr Gln Asn Asn Phe Glu Leu Ala Lys	
85 90 95	

caa att cat gac cag tgg aaa gaa ttt ggc ctg gat ttg gtt gag tta		336	
Gln Ile His Asp Gln Trp Lys Glu Phe Gly Leu Asp Leu Val Glu Leu			
100	105	110	
tcc cat tac gat gtc ttg ctg tcc tat cca aat aaa act cat cct aac		384	
Ser His Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn			
115	120	125	
tat atc tca ata att aat gaa gat gga aat gag att ttc aaa aca tca		432	
Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Lys Thr Ser			
130	135	140	
tta tct gaa cag cca ccc cca gga tat gag aat ata tca gat gta gtg		480	
Leu Ser Glu Gln Pro Pro Gly Tyr Glu Asn Ile Ser Asp Val Val			
145	150	155	160
cca cca tac agt gcc ttc tct cca caa ggg aca cca gag ggt gat cta		528	
Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly Thr Pro Glu Gly Asp Leu			
165	170	175	
gtg tat gtc aac tat gca cga act gaa gac ttc ttt aaa ctg gaa cgg		576	
Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg			
180	185	190	
gaa atg aag atc agt tgt tct ggg aag att gtg att gcc aga tat ggg		624	
Glu Met Lys Ile Ser Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly			
195	200	205	
aaa gtg ttc aga gga aat atg gtt aaa aat gct caa ctg gca ggg gca		672	
Lys Val Phe Arg Gly Asn Met Val Lys Asn Ala Gln Leu Ala Gly Ala			
210	215	220	
aaa gga atg att ctg tac tca gac cct gct gac tac ttt gtt cct gcg		720	
Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Val Pro Ala			
225	230	235	240
gtg aag tcc tat cca gat ggc tgg aac ctc cct gga ggt ggt gtc caa		768	
Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Val Gln			
245	250	255	
cgt gga aat gtc tta aat ctt aat ggt gca ggt gac ccg ctc aca cca		816	
Arg Gly Asn Val Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro			
260	265	270	
ggt tac cca gca aat gaa cat gct tat agg cat gag ttg aca aac gct		864	
Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg His Glu Leu Thr Asn Ala			
275	280	285	
gtt ggc ctt cca agt att cct gtc cat cct att gga tat gat gat gca		912	
Val Gly Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Asp Asp Ala			
290	295	300	

cag aaa ctc tta gaa cac atg ggt cca gca ccc cct gac agt agc Gln Lys Leu Leu Glu His Met Gly Gly Pro Ala Pro Pro Asp Ser Ser 305 310 315 320 960
tgg aag gga gga tta aaa gtg cct tac aac gtg gga cct ggc ttt gct Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Ala 325 330 335 1008
gga aac ttt tca aca caa aag gtc aag atg cat att cac tct tac act Gly Asn Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Tyr Thr 340 345 350 1056
aaa gtg aca aga atc tat aat gtc att ggc acc ctc aaa gga gct ctg Lys Val Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Lys Gly Ala Leu 355 360 365 1104
gaa cca gac aga tat gtt att ctt gga ggt cac cga gac gct tgg gta Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ala Trp Val 370 375 380 1152
ttt ggt ggc att gac cct cag agt gga gca gct gtt gtt cat gaa att Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile 385 390 395 400 1200
gtg cgg agc ttt gga acc ctg aag aag aaa gga cgg agg cct aga agg Val Arg Ser Phe Gly Thr Leu Lys Lys Gly Arg Arg Pro Arg Arg 405 410 415 1248
aca att ttg ttt gca agc tgg gat gca gaa gaa ttt ggc ctt ctt ggt Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly 420 425 430 1296
tct act gag tgg gca gag gaa cat tca aga ctc cta caa gag cga ggt Ser Thr Glu Trp Ala Glu Glu His Ser Arg Leu Leu Gln Glu Arg Gly 435 440 445 1344
gtg gct tat att aat gct gat tct tcc ata gaa gga aat tac act cta Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu 450 455 460 1392
aga gtt gat tgc aca cca ctg atg tac agc tta gtg tac aac cta aca Arg Val Asp Cys Thr Pro Leu Met Tyr Ser Leu Val Tyr Asn Leu Thr 465 470 475 480 1440
aaa gag ctg caa agc cca gat gaa ggt ttt gaa gga aaa tct ctt tat Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr 485 490 495 1488
gac agc tgg aaa gaa aag agt cct tca cct gag ttc att gga atg ccc Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro Glu Phe Ile Gly Met Pro 500 505 510 1536

aga att agc aag ctg ggg tct ggc aat gat ttt gaa gtg ttc ttc caa		1584	
Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln			
515	520	525	
aga ctt gga att gct tca ggc aga gcc cga tat act aaa aat tgg aaa		1632	
Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Lys			
530	535	540	
act aac aaa gtc agc agc tat cct ctc tat cac agt gtc tat gaa aca		1680	
Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr			
545	550	555	560
tat gag ctg gta gta aaa ttt tat gac cca aca ttt aaa tac cac ctc		1728	
Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro Thr Phe Lys Tyr His Leu			
565	570	575	
act gtg gcc cag gtt cga gga gcg atg gta ttt gaa ctt gcc aat tct		1776	
Thr Val Ala Gln Val Arg Gly Ala Met Val Phe Glu Leu Ala Asn Ser			
580	585	590	
ata gtg ctt ccc ttt gac tgc caa agt tat gct gta gct ctg aag aag		1824	
Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr Ala Val Ala Leu Lys Lys			
595	600	605	
tat gct gac act atc tac aat att tca atg aaa cat cca caa gaa atg		1872	
Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met Lys His Pro Gln Glu Met			
610	615	620	
aag gct tac atg ata tca ttt gat tca ctg ttt tct gca gtc aat aat		1920	
Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu Phe Ser Ala Val Asn Asn			
625	630	635	640
ttt aca gat gtt gca tct aag ttc aat cag aga ctg caa gag tta gac		1968	
Phe Thr Asp Val Ala Ser Lys Phe Asn Gln Arg Leu Gln Glu Leu Asp			
645	650	655	
aaa agc aac ccc ata tta ctg aga att atg aat gac cag ctg atg tat		2016	
Lys Ser Asn Pro Ile Leu Leu Arg Ile Met Asn Asp Gln Leu Met Tyr			
660	665	670	
ctg gaa cgt gca ttc att gat cct tta ggc tta cca gga agg cct ttc		2064	
Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Gly Arg Pro Phe			
675	680	685	
tac agg cat acc atc tat gct cca agc agc cac aac aag tat gca gga		2112	
Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly			
690	695	700	
gaa tca ttc cct ggg att tat gat gcc ctt ttt gat ata agt agc aaa		2160	
Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Ser Ser Lys			
705	710	715	720

gtc aat gct tct aag gcc tgg aac gaa gtg aag aga cag att tct att 2208
 Val Asn Ala Ser Lys Ala Trp Asn Glu Val Lys Arg Gln Ile Ser Ile
 725 730 735

gca acc ttt aca gtc gca gca gag act ctg agg gaa gta gct 2256
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 740 745 750

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20 25 30

Thr Phe Leu Ile Gly Phe Leu Phe Gly Trp Phe Ile Lys Pro Ser Asn
35 40 45

Glu Ala Thr Gly Asn Val Ser His Ser Gly Met Lys Lys Glu Phe Leu
50 55 60

His Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr
65 70 75 80

Arg Thr Pro His Leu Ala Gly Thr Gln Asn Asn Phe Glu Leu Ala Lys
85 90 95

Gln Ile His Asp Gln Trp Lys Glu Phe Gly Leu Asp Leu Val Glu Leu
100 105 110

Ser His Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn
115 120 125

Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Lys Thr Ser
130 135 140

Leu Ser Glu Gln Pro Pro Gly Tyr Glu Asn Ile Ser Asp Val Val
145 150 155 160

Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly Thr Pro Glu Gly Asp Leu
165 170 175

Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg
180 185 190

Glu Met Lys Ile Ser Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly
195 200 205

Lys Val Phe Arg Gly Asn Met Val Lys Asn Ala Gln Leu Ala Gly Ala
210 215 220

Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Val Pro Ala
225 230 235 240

Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Val Gln
245 250 255

Arg Gly Asn Val Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro
260 265 270

Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg His Glu Leu Thr Asn Ala
275 280 285

Val Gly Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Asp Asp Ala
290 295 300

Gln Lys Leu Leu Glu His Met Gly Gly Pro Ala Pro Pro Asp Ser Ser
305 310 315 320

Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Ala
325 330 335

Gly Asn Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Tyr Thr
340 345 350

Lys Val Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Lys Gly Ala Leu
355 360 365

Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ala Trp Val
370 375 380

Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile
385 390 395 400

Val Arg Ser Phe Gly Thr Leu Lys Lys Gly Arg Arg Pro Arg Arg
405 410 415

Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly
420 425 430

Ser Thr Glu Trp Ala Glu Glu His Ser Arg Leu Leu Gln Glu Arg Gly
435 440 445

Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu
450 455 460

Arg Val Asp Cys Thr Pro Leu Met Tyr Ser Leu Val Tyr Asn Leu Thr
465 470 475 480

Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr
485 490 495

Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro Glu Phe Ile Gly Met Pro
 500 505 510

Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln
 515 520 525

Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Lys
 530 535 540

Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr
 545 550 555 560

Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro Thr Phe Lys Tyr His Leu
 565 570 575

Thr Val Ala Gln Val Arg Gly Ala Met Val Phe Glu Leu Ala Asn Ser
 580 585 590

Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr Ala Val Ala Leu Lys Lys
 595 600 605

Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met Lys His Pro Gln Glu Met
 610 615 620

Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu Phe Ser Ala Val Asn Asn
 625 630 635 640

Phe Thr Asp Val Ala Ser Lys Phe Asn Gln Arg Leu Gln Glu Leu Asp
 645 650 655

Lys Ser Asn Pro Ile Leu Leu Arg Ile Met Asn Asp Gln Leu Met Tyr
 660 665 670

Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Gly Arg Pro Phe
 675 680 685

Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly
 690 695 700

Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Ser Ser Lys
 705 710 715 720

Val Asn Ala Ser Lys Ala Trp Asn Glu Val Lys Arg Gln Ile Ser Ile
 725 730 735

Ala Thr Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Arg Glu Val Ala
 740 745 750

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<220>

<221> CDS

<222> (1)..(2082)

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ttt tta tac aat ttc aca cgg aca cca cac ttg gca gga aca caa aat	96
Phe Leu Tyr Asn Phe Thr Arg Thr Pro His Leu Ala Gly Thr Gln Asn	
20 25 30	

aat ttt gag ctt gca aag caa att cat gac cag tgg aaa gaa ttt ggc	144
Asn Phe Glu Leu Ala Lys Gln Ile His Asp Gln Trp Lys Glu Phe Gly	
35 40 45	

ctg gat ttg gtt gag tta tcc cat tac gat gtc ttg ctg tcc tat cca	192
Leu Asp Leu Val Glu Leu Ser His Tyr Asp Val Leu Leu Ser Tyr Pro	
50 55 60	

aat aaa act cat cct aac tat atc tca ata att aat gaa gat gga aat	240
Asn Lys Thr His Pro Asn Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn	
65 70 75 80	

gag att ttc aaa aca tca tta tct gaa cag cca ccc cca gga tat gag	288
Glu Ile Phe Lys Thr Ser Leu Ser Glu Gln Pro Pro Pro Gly Tyr Glu	
85 90 95	

aat ata tca gat gta gtg cca cca tac agt gcc ttc tct cca caa ggg	336
Asn Ile Ser Asp Val Val Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly	
100 105 110	

aca cca gag ggt gat cta gtg tat gtc aac tat gca cga act gaa gac	384
Thr Pro Glu Gly Asp Leu Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp	
115 120 125	

ttc ttt aaa ctg gaa cgg gaa atg aag atc agt tgt tct ggg aag att	432
Phe Phe Lys Leu Glu Arg Glu Met Lys Ile Ser Cys Ser Gly Lys Ile	
130 135 140	

gtg att gcc aga tat ggg aaa gtg ttc aga gga aat atg gtt aaa aat	480
Val Ile Ala Arg Tyr Gly Lys Val Phe Arg Gly Asn Met Val Lys Asn	
145 150 155 160	

gct caa ctg gca ggg gca aaa gga atg att ctg tac tca gac cct gct	528
Ala Gln Leu Ala Gly Ala Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala	
165 170 175	

gac tac ttt gtt cct gcg gtg aag tcc tat cca gat ggc tgg aac ctc		576
Asp Tyr Phe Val Pro Ala Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu		
180	185	190
cct gga ggt ggt gtc caa cgt gga aat gtc tta aat ctt aat ggt gca		624
Pro Gly Gly Val Gln Arg Gly Asn Val Leu Asn Leu Asn Gly Ala		
195	200	205
ggt gac ccg ctc aca cca ggt tac cca gca aat gaa cat gct tat agg		672
Gly Asp Pro Leu Thr Pro Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg		
210	215	220
cat gag ttg aca aac gct gtt ggc ctt cca agt att cct gtc cat cct		720
His Glu Leu Thr Asn Ala Val Gly Leu Pro Ser Ile Pro Val His Pro		
225	230	235
240		
att gga tat gat gat gca cag aaa ctc tta gaa cac atg ggt ggt cca		768
Ile Gly Tyr Asp Asp Ala Gln Lys Leu Leu Glu His Met Gly Gly Pro		
245	250	255
gca ccc cct gac agt agc tgg aag gga gga tta aaa gtg cct tac aac		816
Ala Pro Pro Asp Ser Ser Trp Lys Gly Leu Lys Val Pro Tyr Asn		
260	265	270
gtg gga cct ggc ttt gct gga aac ttt tca aca caa aag gtc aag atg		864
Val Gly Pro Gly Phe Ala Gly Asn Phe Ser Thr Gln Lys Val Lys Met		
275	280	285
cat att cac tct tac act aaa gtg aca aga atc tat aat gtc att ggc		912
His Ile His Ser Tyr Thr Lys Val Thr Arg Ile Tyr Asn Val Ile Gly		
290	295	300
acc ctc aaa gga gct ctg gaa cca gac aga tat gtt att ctt gga ggt		960
Thr Leu Lys Gly Ala Leu Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly		
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His Arg Asp Ala Trp Val Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala		
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Ala Val Val His Glu Ile Val Arg Ser Phe Gly Thr Leu Lys Lys Lys		
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gga cgg agg cct aga agg aca att ttg ttt gca agc tgg gat gca gaa		1104
Gly Arg Arg Pro Arg Arg Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu		
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Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp Ala Glu Glu His Ser Arg		
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Val Ile Ala Arg Tyr Gly Lys Val Phe Arg Gly Asn Met Val Lys Asn
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Asp Tyr Phe Val Pro Ala Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu
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 195 200 205

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Ile Gly Tyr Asp Asp Ala Gln Lys Leu Leu Glu His Met Gly Gly Pro
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Pro Asp Thr Arg
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<p>(54) Title: BIOLOGICAL MATERIALS AND METHODS USEFUL IN THE DIAGNOSIS AND TREATMENT OF DISEASES</p> <p>(57) Abstract</p> <p>The present invention relates to a method of making a β-form of a prion protein which preferably has more β-sheet than α-helix structure and is soluble in the absence of a denaturant and/or is non-aggregated and exhibits partial resistance to digestion with proteinase K. The invention also relates to use of the β-form in medicine, especially for raising antibodies useful in the treatment and/or diagnosis of prion diseases. The invention also relates to methods of screening for compounds which are capable of inhibiting and/or reversing the conversion of the native α-form of a prion protein to a β-form, and to uses of identified compounds in medicine.</p>			

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EE	Estonia						

**BIOLOGICAL MATERIALS AND METHODS USEFUL IN THE
DIAGNOSIS AND TREATMENT OF DISEASES**

The present invention relates to prion proteins.

5

Prions are infectious pathogens that differ from bacteria, fungi, parasites, viroids, and viruses, both with respect to their structure and with respect to the diseases that they cause. Molecular biological and structural studies of prions promise to open new vistas into fundamental mechanisms of 10 cellular regulation and homeostasis not previously appreciated. Kuru, Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS) are all human neurodegenerative diseases that are caused by prions and are frequently transmissible to laboratory animals. Familial CJD and GSS are also 15 genetic disorders. No effective therapy exists to prevent these fatal disorders².

In addition to the prion diseases of humans, disorders of animals are included in the group of known prion diseases. Scrapie of sheep and goats 20 is the most studied of the prion diseases. Bovine spongiform encephalopathy (BSE) is thought to result from abnormal feeding practices. BSE threatens the beef industry of Great Britain and possibly other countries; the production of pharmaceuticals involving cattle is also 25 of concern. Control of sheep scrapie in many countries is a persistent and vexing problem².

Since 1986, more than 170,000 cattle have developed BSE in Great Britain. Many investigators contend that BSE, often referred to as "mad cow disease", resulted from the feeding of dietary protein supplements

derived from rendered sheep offal infected with scrapie to cattle, a practice banned since 1988. It is thought that BSE will disappear with the cessation of feeding rendered meat and bone meal, as has been the case in kuru of humans, confined to the Fore region of New Guinea and once the 5 most common cause of death among women and children. Kuru has almost disappeared with the cessation of ritualistic cannibalism.

Prion diseases are associated with the accumulation of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP^{C}) with an increase in its 10 β -sheet content¹. According to the protein-only hypothesis, PrP^{Sc} is the principal or sole component of transmissible prions². Although the structure of PrP^{C} has been determined³ and has been found to consist predominantly of α -helices, the insolubility of PrP^{Sc} , which is isolated from tissue in a highly aggregated state and which has a high β -sheet 15 content, has precluded high-resolution structural analysis. Various workers have attempted to make forms of PrP which are intermediate between the normal (PrP^{C}) form and the abnormal, pathogenic form (PrP^{Sc}), having a predominantly β -sheet form therefore termed the β -form.

20 Hornemann & Glockshuber *PNAS* 95, 6010-6014 (1998)⁸ describe a β -intermediate which is an unfolding intermediate of mouse PrP and contains predominantly β -sheet elements of secondary structure as opposed to α -helix. Swietnicki *et al* (1997) *J. Biol. Chem.* 272:44, Oct 31 pp27517-27520 describe an identical folding intermediate derived from 25 human PrP^{90-231} . The mouse β -intermediate is derived from oxidised PrP which contains the native disulphide bond. The mouse PrP intermediate required urea (a denaturant) for stabilisation. The reference on page 6011 "Results" states that the mouse β -intermediate exhibits stability at pH 4.0 in the absence of denaturant; however this is based upon an equilibrium

calculation. The free energy of folding (Table 1, page 6012) is approximated from a fit of the equation described in Materials and Methods (page 6011) to the data in Figure 1A. From this an equilibrium constant can be calculated which describes the small proportion of molecules that will exist as the β -intermediate in the absence of denaturant. The proportion of molecules in this state is low (around 0.2%) and nothing can be said about their solubility in the absence of denaturant as they are not detectable. Indeed one would argue they are extremely unlikely to be soluble in the absence of denaturant because folding intermediates are structural states that are populated during rearrangement of a polypeptide chain from a random structure to a defined native conformation, or *vice versa*. They are characterised as having native-like secondary structure, few tertiary interactions, increased molecular volume, increased side chain mobility and exposed hydrophobic residues. These properties combined make them prone to aggregation and, as such, are generally insoluble in the absence of denaturants. Several references describe these properties in detail¹⁸⁻²³.

Moreover, the above calculation is dependent upon the transition being a genuine equilibrium, ie. fully reversible. If the transition is not reversible this analysis is invalid. We have performed similar experiments and have found that full reversibility is abolished at protein concentrations in excess of 1 mg/ml, with refolding yields < 100%.

Zhang et al (1997) *Biochem* 36:12, 3543-3553 describe a β -sheet form of recombinant Syrian hamster PrP containing residues 90-231 which is formed by a method involving refolding at a pH of 6.5. It is clear from page 3548, second column and Fig 7, that the β -form described is neither monomeric nor soluble in aqueous solution.

According to a first aspect the invention provides a method of making a β-form of a prion protein which has more β-sheet than α-helix structure, can exist as a monomer and can retain solubility in aqueous solution in the
5 absence of a denaturant, the method comprising:

providing a reduced prion protein which does not include a disulphide bond and causing the conformation of the protein to change so that it adopts the β-form.

10 Preferably, the change in conformation is caused by exposure to conditions of acidic pH, preferably a pH of 5.5 or less, more preferably a pH of 4.8 or less and most preferably a pH of 4.0.

Skilled persons will appreciate that the β-sheet and α-helix structure can
15 be shown by circular dichroism spectropolarimetry as described herein. While the native prion protein state is characterised by a strong α-helical signal, the β-form of the invention shows a shift to a conformation dominated by β-sheet. By “dominated” in this context we include the meaning that there is more β-sheet structure of the prion protein than α-
20 helix structure.

By “exist as a monomer” we include the meaning that the β-form of the prion protein does not exist as an aggregate of two or more β-form prion proteins. Skilled persons will appreciate that analytical sedimentation
25 studies can be used to determine whether or not a protein exists in solution as a monomer or as an aggregate of two or more proteins. A suitable technique is described in Zhang et al (1997) Biochem, 36:12, 3542-3553 (see page 3545-3546 passage entitled Analytical Sedimentation). The

technique involves the use of an analytical ultracentrifuge (Beckman Optimat XL-A) equipped with a six channel cell, using ultraviolet absorption between 220 and 280nm.

5 By "can retain solubility in the absence of a denaturant" we include the meaning that a significant proportion eg around 30% or more of the β-form remains in solution as a monomer after centrifugation at 100,000 g for 1 hour and preferably 150,000 g for 8-16 hours, most preferably at 200,000 g for 8-16 hours. The centrifugation may be carried out on a 2
10 mg/ml aqueous solution of the β-form prion protein comprising Na Acetate + 10mM Tris. HCl + pH 4.0 at 25°C. The structural characteristics of the remaining protein in solution can be determined by circular dichroism spectropolarimetry, for example.

15 Preferably, the β-form remains soluble without denaturant to a concentration of more than 1 mg/ml, more preferably at least 12 mg/ml, and especially more than 20 mg/ml.

It will of course be appreciated that the above requirement for the β-form
20 to *be capable of* retaining solubility in the absence of the denaturant in no way limits the invention to methods or compositions which do not include a denaturant.

A β-form of a prion protein of the invention also comprises a prion
25 protein which has at least 20% of its residues in β-sheet structure, more preferably at least 50% and most preferably 50 to 60% or more, as determined by CD spectropolarimetry.

A β -form of a prion protein of the invention also comprises a prion protein which is non-aggregated and exhibits partial resistance to proteinase K digestion.

5 A β -form of a prion protein of the invention also comprises a prion protein which is non-aggregated but is capable of forming an aggregated fibrous and/or amyloid form, preferably on exposure to a denaturant.

Preferably, a β -form of a prion protein of the invention also comprises a
10 prion protein which is non-aggregated but is capable of forming a non-fibrillar aggregate on exposure to conditions of sufficient ionic strength. Preferably, the non-fibrillar aggregate is capable of forming a fibrillar structure.

15 By "conditions of sufficient ionic strength" we mean an ionic strength capable of converting the non-aggregated β -form to an aggregated form. For example, salt concentrations of 50 mM to 500 mM, especially 100 mM or more are sufficient to cause murine β -form prion protein to form a non-fibrillar aggregate. A particularly preferred salt concentration is 100
20 – 200, more preferably 150 mM eg NaCl or KCl.

A β -form of a prion protein of the invention also comprises a prion protein which is capable of interconverting between a β -form as defined herein and an α -form of a prion protein as described herein.

25

A β -form of a prion protein of the invention may exhibit one or more of the above properties.

In another aspect, the invention provides a method of obtaining non-aggregated β -form from a sample comprising partially digesting the sample with proteinase K.

5 It will be appreciated that by "prion protein" is included variants, fragments and fusions that have interactions or activities which are substantially the same as those of a full length prion protein sequence, but which may be more convenient to use, for example in an assay. A "variant" will have a region which has at least 70% (preferably 80, 90, 95
10 or 99%) sequence identity with the 91-231 region of native human PrP sequence described herein or the corresponding region in the PrP of other species as measured by the Bestfit Program of the Wisconsin Sequence Analysis Package, version 8 for Unix. The percentage identity may be calculated by reference to a region of at least 50 amino acids (preferably at
15 least 75, 100, 120 or 140) of the candidate variant molecule, and the most similar region of equivalent length in the native 91-231 region, allowing gaps of up to 5%.

The percent identity may be determined, for example, by comparing
20 sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Neddeleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith
25 and Waterman (*Adv. Appl. Math.* 2.482, 1981). The preferred default parameters for the GAP program include : (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Bribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986 as described by Schwartz and

Dayhoff, eds, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

5

Hybrid prion proteins comprising amino acid sequences from two or more different species also fall within the scope of the term "prion protein" used herein. Hybrid proteins comprising protein domains from different species can be produced using known recombinant DNA techniques, such 10 as those described in WO93/20093 in relation to hybrid human/porcine factor VIII proteins.

A "fragment" comprises at least 50, preferably 75, 100, 120 or 130 amino acids of the native 91-231 sequence.

15

Such activities will include the abilities mentioned herein, such as the ability to be soluble without denaturant and may include the ability to raise antibodies and for use in screening compounds in accordance with the following aspects of the invention and the ability to form an aggregated 20 fibrous and/or amyloid form especially a non-fibrillar aggregate which preferably comprises spherical particles having a diameter of from approx 10 - 20 nM which can be visualised by electron microscopy, when exposed to suitable conditions etc.

25 Preferably, the β -form of a prion protein exhibits partial resistance to digestion with proteinase K(PK).

By "partial resistance to digestion with proteinase K (PK)" we include the meaning that after incubation of 1 mg/ml of the protein in 10mM

NaAcetate + 10mM Tris. Acetate, pH 8.0 with 0.5 µg/ml PK (based on the total digestion reaction volume) at 37°C for 30 mins some protein can be shown to be undigested when subjected to SDS-PAGE as described herein. Preferably, the majority of the protein is undigested.

5

Preferably, the β-form of the invention displays resistance to digestion at increased concentrations of PK eg 5 µg/ml PK or more.

The disease-related isoform of PrP, PrP^{Sc}, is distinguished biochemically
10 from the normal cellular isoform of the protein, PrP^c, by its partial resistance to digestion with the enzyme proteinase K. We have now demonstrated that not only aggregated β-PrP is protease resistant but also that the soluble β-PrP monomer is also PK-resistant and to a level approximating to that seen with PrP^{Sc}. This is strong evidence to support
15 the contention that β-PrP may be the precursor of PrP^{Sc}.

The novel β-form, or an aggregate of two or more β-forms, of the invention may be used to prepare antibodies which selectively recognise the β-form (whether aggregated or not) rather than the α-form or *vice*
20 *versa*.

By “α-form” of a prion protein we include the meaning of a prion protein which has more α-helical than β-sheet structure. The α-form may also be characterised by sensitivity to degradation by proteinase K.

25

Any reductant and conditions which allow reduction can be used in the method of the invention as long as they do not cause irreversible modification to the polypeptide chain. Reduction of a disulphide bond can

be determined by Ellman's assay (Ellman, G. L., 1959, *Arch Biochem & Biophys*). Reduction of the disulphide bond preferably takes place before the pH is lowered. The acidic pH at which conformation change takes place may be approximately pH 5.5 or less, and preferably pH 4.8 or less,
5 most preferably a pH of 4.0. Skilled persons will appreciate that any buffer that is effective around pH 4.0 can be used, such as 10mM NaAcetate + 10mM Tris.Aacetate.

Preferably, the β -form has substantially the same molecular volume
10 (measured by size exclusion chromatography) as the native form of the prion protein.

In a second aspect, the invention provides a preparation of a β -form of a prion protein wherein at least 1% of the β -form can exist as a monomer
15 and can retain solubility in aqueous solution in the absence of a denaturant. Preferably, the β -form is obtainable by a method according to the first aspect of the invention.

The invention also provides the above (soluble, undenatured) β -form of a prion protein for use in medicine, preferably in the prevention, treatment
20 and/or diagnosis of a prion disease.

It will be appreciated that by virtue of properties such as its solubility, the β -form is amenable to high resolution structural analysis and so has
25 particular utility for research into the mechanisms of prion disease especially prion replication. Such utility is not found in known insoluble forms of prion proteins.

The prion disease may be selected from one or more of the diseases affecting humans. Alternatively or additionally, the prion diseases are selected from one or more of the diseases which affect domestic farm animals such as cows, sheep and goats. Other prion diseases include
5 transmissible mink encephalopathy; chronic wasting disease of mule deer and elk, bovine spongiform encephalopathy and, more recently, a whole series of new animal diseases that are thought to have arisen from their dietary exposure to the BSE agent. These include feline spongiform encephalopathy, affecting domestic cats and captive wild cats (such as
10 cheetahs, pumas, ocelots, tigers) and spongiform encephalopathies of captive exotic ungulates (including kudu, nyala, gemsbok, eland).

Preferably, the prion protein is selected from human, bovine or ovine prion proteins, more preferably human prion protein.

15

According to a third aspect of the invention there is provided a method of making an antibody against a prion protein having a β -form as defined in accordance with the earlier aspects of the invention, comprising administering said β -form to an animal and collecting and purifying the
20 directly or indirectly resulting antibody. The antibody may be polyclonal, but is preferably monoclonal.

By "antibody" in accordance with the invention we include molecules which comprise or consists of antigen binding fragments of an antibody
25 including Fab, Fv, ScFv and dAb. We also include agents which incorporate such fragments as portions for targetting prion molecules and/or cells or viruses which display such molecules.

According to this aspect of the invention, there is also provided a monoclonal antibody capable of distinguishing between the native α -form and the β -form of a prion protein as defined in accordance with earlier aspects of the invention or *vice versa*. Also provided is a hybridoma cell
5 capable of producing such a monoclonal antibody.

In accordance with this aspect of the invention there is also provided an antibody for use in medicine, which antibody binds preferentially to the β -form of a prion protein rather than to the α -form of the prion protein or
10 *vice versa*. Preferably, the antibody is for use in the manufacture of a composition for use in the prevention, treatment and/or diagnosis of a prion disease.

According to a fourth aspect of the invention there is provided a method of
15 detecting the presence of a prion protein having a β -form as defined in accordance with the earlier aspects of the invention in a biological sample. The method preferably comprises providing an antibody preparation comprising an antibody which preferentially binds the β -form rather than the α -form and detecting whether the antibody binds β -form.
20

Conveniently, the antibody is directly or indirectly labelled by suitable means and its binding to the β -form is detected by detecting a label.

Preferably, the biological sample comprises or consists of a bodily fluid or
25 tissue such as blood or blood derivative, ie a component such as plasma, lymphoid tissue (such as tonsils, appendices, lymph or spleen), cerebrospinal fluid faeces, urine, lymph or sputum. The biological sample may be a tissue sample eg a biopsy tissue sample.

It may be advantageous to introduce an anti- β -form antibody into one of the tissues mentioned above either to detect β -form or to remove β -form before it reaches the brain. Such anti- β -form antibodies are preferably 5 antibodies which preferentially react with the β -form rather than the normal α -form of the prion protein.

By "preferentially" according to the various aspects of the invention we include the meaning that the ratio of α/β binding may be 45/55, 25/75, 10 more preferably, 10/90, 5/95, 1/99 or substantially 0/100.

The invention also provides a method of detecting antibodies in a biological sample, which antibodies bind preferentially to a β -form of a prion protein rather than the α -form comprising exposing the β -form to the biological 15 sample to permit binding of antibody to the β -form and detecting the binding of antibody to the β -form. Optionally, the β -form is immobilised before exposure to the sample.

The invention also provides a method of obtaining a β -form binding agent 20 which binds preferentially to a β -form of a prion protein rather than an α -form comprising exposing the β -form to a sample to permit binding of agents to the β -form and optionally collecting the agent bound to the β -form. Optionally, the β -form is immobilised before exposure to the sample. Preferably, the binding agent is directly or indirectly labelled and its binding 25 to the β -form is detected by detecting the label.

The invention also provides a kit useful for diagnosing a prion disease from a biological sample comprising a binding agent, preferably an antibody,

which is capable of preferentially binding the β -form rather than the α -form, or a β -form of a prion protein which binds said binding agent; and means for detecting binding of the binding agent to the β -form. The binding agent or β -form being coupled optionally to an inert support. Preferably, 5 the means for detecting binding comprises a radioactive, enzymic or fluorescent label.

The invention also provides an *in vitro* method for diagnosing a predisposition to, or the presence of, a prion disease comprising providing 10 a reduced α -form of a prion protein, preferably at a pH of around 5.5 or less, preferably pH 4.8 or less, most preferably a pH of 4.0; comparing the amount or rate of formation of a β -form as defined herein in the presence and absence of a biological sample eg from a patient. Increased 15 rate or amount of β -form formation is indicative of a predisposition to, or the presence of, a prion disease.

The invention also provides a method of treating a biological sample to remove a β -form of a prion protein comprising providing a binding agent which binds preferentially to the β -form of a prion protein rather than to 20 the α -form of the prion protein, exposing the biological sample to the binding agent whereby a β -form of a prion protein can bind the binding agent and optionally collecting the treated biological sample. Preferably, the binding agent is immobilised before the exposure to the sample.

25 The invention also provides a method of diagnosing a predisposition to, or the presence of, a prion disease comprising providing a β -form of a prion protein; providing a biological sample; and exposing the solution to the sample and detecting the presence of an aggregation of the β -form, such an

aggregation being indicative of predisposition to, or the presence of, a prion disease.

Preferably, the aggregation of the β -form is a non-fibrillar aggregate
5 which preferably comprises spherical or irregularly shaped particles having a diameter of from 10-20 nm which can be visualised by electron microscopy.

The invention also provides the use of a β -form or a non-fibrillar aggregate
10 thereof in the manufacture of a composition for use as a vaccine against a prion disease. A vaccine composition of the invention preferably comprises a β -form or a non-fibrillar aggregate thereof and an adjuvant.

According to a fifth aspect of the invention there is provided a method of
15 identifying an agent that is capable of preventing, reducing and/or reversing the conversion of a prion protein to a β -form as defined above, the method comprising: providing a sample of a prion protein and comparing the amount of the β -form quantitatively or qualitatively in the presence and absence of a test agent.

20

In a sixth aspect of the invention, there is provided a method of identifying an agent that is capable of preventing or reducing the conversion of a prion protein from the β -form, as defined in accordance with earlier aspects of the invention, to an aggregated fibrous and/or amyloid form,
25 especially a non-fibrillar aggregate mentioned above, the method comprising providing a solution containing the β -form and comparing qualitatively or quantitatively the amount of the aggregated and/or amyloid form produced in the presence and absence of a test agent.

Preferably, the amount of the aggregated and/or amyloid, especially non-fibrillar aggregate, form is measured using a spectrofluorimeter.

In a seventh aspect of the invention there is provided an agent which is
5 identifiable by a method as defined in accordance with the fifth or sixth
aspect of the invention.

In an eighth aspect the invention provides an agent capable of preventing,
reducing and/or reversing the conversion of a prion protein from an α -
10 form to a β -form as defined in accordance with earlier aspects of the
invention.

In a ninth aspect the invention provides an agent capable of preventing or
reducing the conversion of a β -form of a prion protein as defined in
15 accordance with earlier aspects of the invention to an aggregated and/or
amyloid , especially non-fibrillar aggregate, form.

The agents according to the seventh, eighth and ninth aspects of the
invention may be a drug-like compound or lead compound for the
20 development of a drug-like compound. Thus, the methods may be
methods for identifying a drug-like compound or lead compound for the
development of a drug-like compound that is capable of preventing,
reducing and/or reversing the conversion of a prion protein to a β -form;
and/or that is capable of preventing and/or reducing the conversion of the
25 β -form to an aggregated and/or amyloid, especially non-fibrillar
aggregate, form.

The term “drug-like compound” is well known to those skilled in the art,
and may include the meaning of a compound that has characteristics that

may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or 5 biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons molecular weight and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that 10 these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent 15 against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise, too toxic or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

20 The compounds identified in the methods of the invention may themselves be useful as a drug or they may represent lead compounds for the design and synthesis of more efficacious compounds.

In another aspect the invention provides an agent that comprises a binding 25 agent portion which binds preferentially to the β -form of the prion protein rather than the α -form, and an effector portion which is capable of one or more of the following functions: (1) preventing, reducing and/or reversing the conversion of a prion protein to a β -form; (2) preventing or reducing the conversion of a prion protein from the β -form to an aggregated fibrous

and/or amyloid, especially a non-fibrillar aggregate form; or (3) destroying a β -form of a prion protein and/or a cell or virus displaying such a protein.

5 Preferably, the binding agent portion comprise an antibody or a fragment thereof. Preferably the antibody or fragment thereof is made according to aspects of the present invention.

In one preferred embodiment the effector portion of an agent comprises a
10 compound of the earlier aspects of the invention.

In another preferred embodiment the agent comprises an effector portion which is directly or indirectly cytotoxic.

15 By a "directly cytotoxic" portion we include a portion of an agent which is in itself toxic to the cell if it reaches, and preferably enters, the said cell.

By an "indirectly cytotoxic" portion we include a portion of an agent
20 which can be converted into or produce a cytotoxic agent by the action of a further reagent, or which can convert a substantially non-toxic substance into a toxic substance. We also include a portion of an agent which can bind specifically to a compound which is directly or indirectly cytotoxic.

25 Non-limiting examples of cytotoxic portions include a drug, pro-drug, radionuclide, protein including an enzyme, antibody or any other therapeutically useful reagent, including cytokines such as tumour necrosis factor, interleukin-2 or interferon- γ .

Thus, the drug may be a cytotoxic chemical compound such as methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), daunorubicin or other intercalating agents. The protein may be ricin. The cytotoxic portion may comprise a highly radioactive atom,
5 such iodine-131, rhenium-186, rhenium-188 or yttrium-90.

The enzyme, or enzymatic portion thereof, may be directly cytotoxic, such as DNaseI or RNase, or indirectly cytotoxic such as an enzyme which converts a substantially non-toxic pro-drug into a toxic form. The
10 enzyme cytosine deaminase converts 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) (Mullen *et al* (1922) *PNAS* 89, 33); the herpes simplex enzyme thymidine kinase sensitises cells to treatment with the antiviral agent ganciclovir (GCV) or aciclovir (Moolten (1986) *Cancer Res.* 46, 5276; Ezzedine *et al* (1991) *New Biol* 3, 608). The cytosine deaminase of
15 any organism, for example *E. coli* or *Saccharomyces cerevisiae*, may be used. Examples of the construction of antibody-enzyme fusions are disclosed by Neuberger *et al* (1984) *Nature* 312, 604.

Other examples of pro-drug/enzyme combinations include those disclosed
20 by Bagshawe *et al* (WO 88/07378), namely various alkylating agents and the *Pseudomonas* spp. CPG2 enzyme, and those disclosed by Epenetos & Rowlinson-Busza (WO 91/11201), namely cyanogenic pro-drugs (for example amygdalin) and plant-derived α -glucosidases. The nitroreductase/CB1954 system described by Bridgewater *et al* (1995) *Eur. J. Cancer* 31A, 2362-2370 is another example of an enzyme/prodrug combination suitable for use in the invention.
25

In a tenth aspect the invention provides an agent in accordance with the earlier aspects of the invention for use in medicine. Preferably, use of the

aspects in the manufacture of a composition for use in the prevention, treatment and/or diagnosis of a prion disease, or for use as a research reagent.

- 5 In an eleventh aspect the invention provides a pharmaceutical composition comprising a pharmaceutically effective amount of an agent in accordance with the seventh, eighth and/or ninth aspects of the invention, together with a pharmaceutically acceptable diluent or carrier.
- 10 In a twelfth aspect the invention provides a method of preventing and/or treating a prion disease comprising administering to a subject an effective amount of an agent in accordance with the earlier aspects of the invention.

By “effective amount” we include the meaning that sufficient quantities of
15 the agent are provided to produce a desired pharmaceutical effect
beneficial to the health of the recipient.

For a better understanding, the following non-limiting examples which
embody certain aspects of the invention will now be described with
20 reference to the following figures.

Figure 1

- (a) Secondary and tertiary structure of the two human PrP isoforms.
- 25 The main graph shows CD spectra collected in the far UV region. Oxidised human PrP at pH 8.0 is shown in open circles and displays a typically α -helical spectrum with 47% of amide residues involved in helical structure¹⁷. In contrast reduced human PrP at pH 4.0 displays a β -sheet spectrum, shown in open triangles. There is little or no helix

present with up to 40% of amide residues adopting a β -sheet conformation¹⁸. The inset displays near UV CD spectra for oxidised human PrP pH 8.0 (open circles), reduced human PrP pH 4.0 (open triangles) and denatured human PrP (open squares). The oxidised protein
5 clearly displays a high level of tertiary organisation in the aromatic region of the spectrum, whereas the denatured PrP lacks any distinct tertiary interactions. The reduced human PrP displays a level of tertiary organisation intermediate between native and denatured states.

10 (b) ^1H NMR spectra of the upfield regions of the α - and β -forms of huPrP⁹¹⁻²³¹. Peaks upfield of 0.7ppm are characteristic of strong tertiary interactions between methyl groups and aromatic rings found in folded, globular proteins.

15 (c) Expanded region of a ^1H , ^{15}N HSQC spectrum of the β -form of huPrP⁹¹⁻²³¹ showing its chemical shift dispersion, which is much reduced relative to the α -form (Hornemann S. and Glockshuber R., *J Mol Biol*, **261**, 614-619 (1996)).

20 While the 1D ^1H -NMR spectrum of native human PrP⁹¹⁻²³¹ exhibits wide chemical shift dispersion characteristic of a fully folded globular protein, the 1D ^1H and ^1H ^{15}N HSQC spectra of the β -form of PrP exhibit considerably less chemical shift dispersion (Fig 1b,c). This lack of dispersion is characteristic of the loss of fixed side chain interactions,
25 which, in conjunction with the aromatic CD results, suggests some similarities with molten globule states. In addition, proton and nitrogen line-widths of the β -form (Fig 1c) are comparable to those observed in the folded and unfolded regions of the α -PrP conformation indicating that the

β -form is monomeric at the extremely high concentrations required for NMR, thus confirming the gel-filtration results. The mobile unstructured regions of β -PrP have been assigned from the sharpness and height of the peaks. We find that residues 91-126 and 229-230 are mobile in β -PrP,
5 moreover, this is the same region that is unstructured in the α -PrP conformation. Hence, the rearrangement from α -helix to β -sheet must occur within the structured region of the cellular conformation.

Figure 2

10

Determination of the apparent molecular weight of PrP by size exclusion chromatography.

(a) Elution profile of molecular weight standards used to construct a
15 calibration curve of molecular weight versus elution time (not shown). (b) Oxidised human PrP pH 8.0 in the alpha form elutes with an apparent molecular weight of 18 kDa. This excess weight (calculated mass is 16248 kDa) is due to the large molecular volume of PrP resulting from the dispersed secondary structure elements. (c) Reduced human PrP pH 4.0
20 in the β -form also elutes as a monomer with an apparent molecular weight of 18 kDa. (d) Oxidised human PrP at pH 4.0 partially denatured with 1M GuHCl. Addition of 1M GuHCl to oxidised human PrP at pH 4.0 results in aggregation and precipitation. Clarified supernatant contains a
25 denatured form of PrP with an increased molecular volume corresponding to an apparent molecular weight of 40 kDa.

Figure 3

β -PrP is more prone to form high molecular weight aggregates than α -PrP. Right angle light scattering of a 1 mg/ml solution of α -PrP (open circles) shows there are no high molecular weight aggregates formed upon addition of GuHCl. In contrast β -PrP, which is highly soluble in aqueous buffer alone, readily forms high molecular weight aggregates upon the addition of low concentration of GuHCl (open triangles). Maximum precipitation occurs at 0.4 M GuHCl, with subsequent re-dissolution of aggregates at higher concentrations of denaturant.

Figure 4

β -PrP aggregates self-assemble into fibrils. The protein aggregates appear in two forms by negative stain electron microscopy. (A) The most common form is small (about 10 nm diameter) irregularly shaped and is seen in all samples. (B) The other aggregation form is fibrils which are increasingly prevalent the longer the sample is incubated. These fibres can be seen to intertwine, again a phenomenon that increases with time. Scale bars shown in white represent a length of 200 nm. In order to comply with safety regulations governing the handling of prion protein, electron microscopy was performed on mouse PrP⁹¹⁻²³¹ treated in an identical manner to the human protein.

β -PrP, at a concentration of 0.27 mg/ml in 20 mM sodium acetate pH4, was treated with 1/9 volumes of a 5M stock of GuHCl to give a final protein and denaturant concentrations of 0.25 mg/ml and 0.5 M respectively. The procedure for staining the protein is as follows. A

dilute solution of PrP (~2 μ l) is dropped onto the grid and the molecules adhere to the carbon film. Bonding to the surface prevents interactions between protein molecules. The sample is then flooded with 2% uranyl acetate w/v which coats the carbon surface and any particles stuck to it.

5 The excess is blotted off leaving a thin film. This procedure seldom, if ever, leads to aggregation owing to the initial adherence to the grid surface. In our hands, when doing extensive single molecule work, we have not seen aggregation phenomena using this method. Further, when the PrP molecule is initially laid down the particles are small and circular

10 and only produce fibrils after several hours. If the laying down process caused the aggregation we would not see this time-dependent behaviour.

Figure 5

15 β -PrP displays partial PK resistance in monomeric and aggregated states. α -PrP is sensitive to PK digestion and is completely digested at 0.5 μ g/ml PK. The concentrations of PK indicated are the final concentrations in the digestion reactions.

20 Using identical conditions for digestion in which β -PrP remains soluble and monomeric (data not shown), soluble β -PrP has partial resistance to proteinase K with the majority of protein undigested at 0.5 μ g/ml. Aggregated β -PrP possesses increased resistance to PK digestion with some protein surviving intact at 5 μ g/ml PK. The concentrations of PK indicated are the final concentrations in the digestion reactions. Although β -PrP reverts to α -PrP at pH8.0 this process requires several days for completion. Within the timescale of PK digestion the protein remains as β -PrP.

Figure 6

Known prion protein sequences from other mammalian species, using the
5 single letter code for amino acids as follows:

A=Ala; D=Asp; E=Glu, F=Phe; K=Lys; L=Leu; M=Met;
N=Asn; P=Pro; Q=Gly; R=Arg; S=Ser; T=Thr; and V=Val.

10 Such information is available from databases such as EMBL, Genbank,
Swis-Prot, Brookhaven.

METHODS

15 **1. Purification of human PrP**

Plasmid Design and Protein Expression

The open reading frame of the human PrP gene was amplified by PCR
20 using oligonucleotide primers designed to create an unique N-terminal
BamHI site and C-terminal HindIII site for directional cloning of the
fragment into the expression vector pTrcHisB (Invitrogen Corp.). The
primer corresponding to the N-terminal region of *PRNP* to be expressed
was designed to mutate a glycine at codon 90 to methionine, with the C-
25 terminal primer replacing a methionine residue at 232 to a stop codon.

Human PrP open reading frame.

1 ATGGCGAACCTTGGCTGCTG GATGCTGGTT CTCTTGTGG CCACATGGAG
 51 TGACCTGGGCCTCTGCAAGA AGCGCCCGAA GCCTGGAGGA TGGAACACTG
 101 GGGGCAGCCGATACCCGGGG CAGGGCAGCCCTGGAGGCCA CCGCTACCCA
 151 CCTCAGGGCG GTGGTGGCTGGGGCAGCCTCATGGTGGTG GCTGGGGCA
 201 GCCTCATGGTGGTGGCTGGGGCAGCCCCATGGTGGTG GCTGGGGCA
 251 CTCATGGTGGTGGCTGGGGTCAAGGAGGTG GCACCCACAG TCAGTGGAAC
 301 AAGCCGAGTA AGCCAAAAAC CAACATGAAGCACATGGCTGGCTGCAGC
 351 AGCTGGGGCA GTGGTGGGGGCCTTGGCGCTACATGCTGGAGTGCACCA
 401 TGAGCAGGCCCATCATACTTTCGGCAGTG ACTATGAGGA CCGTTACTAT
 451 CGTGAAAACA TGCACCGTTACCCAAACCAAGTGTACTACA GGCCCATTGGA
 501 TGAGTACAGCAACCAGAACAACTTTGTGCA CGACTGCGTC AATATCACAA
 551 TCAAGCAGCACCGTCACC ACAACCACCA AGGGGGAGAA CTTCACCGAG
 601 ACCGACGTTAAGATGATGGA GCGCGTGGTTGAGCAGATGT GTATCACCA
 651 GTACGAGAGGAATCTCAGGCCTATTACCA GAGAGGATCG AGCATGGTCC
 701 TCTTCTCCTCTCCACCTGTGATCCTCCTGA TCTCTTCCTCATCTCCTG
 751 ATAGTGGGATGA

PCR primers for creation of PrP⁹¹⁻²³¹

N-terminal sense oligo :

5' - T TTG GAT CCG ATG CAA GGA GGT GGC ACC CAC - 3'

C-terminal antisense oligo :

5' - CAA GAA GCT TTC AGC TCG ATC CTC TCT GG - 3'

The ligated pTrcHisB/*PRNP* construct was used to transform the *E. coli* host strain BL21 (DE3) (Novagen), genotype F' *ompT hsdS_B (r_Bm_B) gal dcm* (DE3) which was then plated onto Luria-Bertoni (LB) agar plates containing 100µg/ml carbenicillin. Following growth overnight at 37°C
5 single colonies were picked and used to inoculate 10 x 10ml of LB broth containing 100µg/ml carbenicillin. This culture was grown overnight at 37°C with vigorous shaking. The 10ml cultures were used as inocula for 10 x 1 litre of LB broth containing 100µg/ml carbenicillin which had been pre-warmed to 37°C. Growth at 37°C with vigorous shaking was allowed
10 to progress until the culture reached an OD₆₀₀ of 0.6. Expression was then induced by addition of isopropyl-β-D-galactopyranoside to a final concentration of 1mM and the culture resupplemented with carbenicillin to a level of 100µg/ml. Following 4 hours of induced growth the cells were harvested by centrifugation at 8,500 rpm for 10 minutes.

15

Extraction, Refolding and Purification of Recombinant Human PrP

The cell pellet was resuspended in 50ml of lysis buffer (50mM Tris. Cl pH 8.0, 200mM NaCl, 0.1% Triton X100, 10µg/ml DNase 1, 10µg/ml
20 lysozyme) and disrupted by sonication in 1 minute bursts for a total of 5 minutes. Centrifugation at 9,600 rpm for 30 minutes pelleted all the insoluble material and the supernatant was discarded. The pellet was then washed twice by resuspension in 50ml of lysis buffer with centrifugation at 7,500 rpm for 5 minutes between each wash. Solubilisation of protein
25 in the pellet was performed by resuspension in 50ml of 50mM Tris. Cl, 6M GuHCl, 100mM DTT pH 8.0. Cell debris and insoluble material was removed by centrifugation at 9,600rpm for 30 minutes. The supernatant was clarified by passage through a 0.2µm filter and loaded onto a 20ml

Ni-NTA-Sepharose (Quiagen) column pre-equilibrated with 50mM Tris.Cl, 6M GuHCl pH 8.0.

After washing the column with the above buffer, bound protein was eluted
5 with a 15 column volume linear gradient of 0mM to 300mM imidazole in loading buffer. Recombinant PrP eluted at 185mM imidazole. Eluted fractions were pooled and oxidation of disulphides was achieved by vigorous stirring in the presence of 1 μ M CuSO₄ and dissolved atmospheric oxygen for 16 hours. PrP containing oxidised disulphides was separated
10 from reduced protein using reverse phase chromatography on an RP304-C4 column. The protein was loaded in 50mM Tris.Cl, 6M GuHCl pH 8.0, washed with ddH₂O + 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 15% to 60% acetonitrile + 0.09% TFA. Human
PrP emerged as two major peaks; oxidised protein at 40% acetonitrile and
15 a second peak containing reduced PrP eluted at 45% acetonitrile. The oxidised peak fractions were pooled and neutralised by the addition of 1M Tris.Cl pH 8.0 to a final concentration of 100mM and saturated ammonium sulphate added to a final concentration of 70%. Precipitated
PrP accumulated at the interface between organic and aqueous phases and
20 was removed to a separate container. The protein was solubilised in a minimal volume of 50mM Tris.Cl, 6M GuHCl pH 8.0 and then diluted rapidly to a protein concentration of 1mg/ml and dialysed for 16 hours against 50mM Tris.Cl pH 8.0 with a buffer change after 8 hours. Following dialysis the N-terminal fusion peptide was removed by addition
25 of enterokinase at 1unit/3mg protein. Cleavage was allowed to occur at 37°C for 14 hours and terminated by the addition of "protease complete" (Boehringer Mannheim Corp).

Final purification was carried out by applying the protein material to a 10ml S-Sepharose FastFlow column equilibrated with 25mM Tris.Cl pH 7.0 and following a 5 column volume wash with the same buffer, protein was eluted with a 10 column volume linear gradient of 0mM to 5 300mM NaCl. Recombinant PrP lacking the N-terminal fusion peptide eluted at 150mM whilst uncleaved material remained bound until 250mM NaCl. Eluted fractions were concentrated in an Amicon cell with a 10kDa cut off membrane and then dialysed overnight against 25mM Tris.Cl pH 7.0, 0.02% NaAzide containing a small amount of activated charcoal.

10 Sucrose was added to 5% w/v and the protein snap frozen in liquid nitrogen for long term storage at -80°C.

Recombinant human PrP in the oxidised α -form was purified as described above and dialysed into 10mM NaAcetate + 10mM Tris.HCl pH 8.0. To 15 convert this material to the β -form the protein was reduced and denatured in 100mM DTT in 6M GuHCl + 10mM NaAcetate + 10mM Tris.HCl pH 8.0 for 16 hrs. The protein was refolded by dialysis against 10mM NaAcetate + 10mM Tris.HCl + 1mM DTT pH4.0 and precipitated material removed by centrifugation at 150,000g for 8 hrs. Protein 20 concentration was determined by UV absorption using a calculated molar extinction coefficient of 19632 M⁻¹ cm⁻¹ at 280 nm.

2. *Determination of aggregation state of PrP by gel filtration*

25 A Bio-Sil 125-5 size exclusion column (BioRad) was equilibrated with the appropriate buffer at a flow rate of 1ml/min producing a back pressure of 900 psi. A 20 μ l (360 μ g) aliquot of molecular weight standards (BioRad) containing markers of 670 kDa, 158 kDa, 44 kDa, 17 kDa and 1.35 kDa was loaded onto the column equilibrated with 10 mM NaAcetate +

10 mM Tris.HC1 + 50 mM NaCl. The markers were eluted with 2 column volumes (30 ml) of the same buffer and used to construct a calibration curve for the column. The α -PrP was loaded in a volume of 100 μ l (200 μ g) and eluted with 30 mls of 10 mM NaAcetate +
5 10 mM Tris.HC1 + 50 mM NaCl pH 8.0. β -PrP was loaded in volume of 100 μ l (200 μ g) and eluted with 30 mls of Na Acetate + 100 mM Tris.HC1 + 50 mM NaCl pH 4.0.

3. Circular dichroism spectropolarimetry

10

For circular dichroism (CD) measurements 62.5 μ M protein was incubated at 10 mM NaAcetate + 10 mM Tris.HC1 at either pH 8.0 (α -PrP) or pH 4.0 (β -PrP) and molecular ellipticity ([θ], degree M⁻¹ cm⁻¹) was recorded in the far UV range between 190 nm and 250 nm, using a
15 xenon light source in a Jobin-Yvon CD6 spectrometer (cell path length 0.01 cm, slit width 1.0 nm; 2 nm bandwidth, integration time 20 sec). Near UV CD spectra were recorded between 250 nm and 310 nm using 62.5 μ M protein in a 10 nm pathlength cuvette with a slit width of 1.0 nm (2 nm bandwidth, integration time 20 sec). All data were recorded at
20 25°C.

4. NMR Spectroscopy

NMR spectra shown were acquired at 293 K on a Bruker DRX-500
25 spectrometer. Sample conditions were as follows, α -form : 1 mM human PrP⁹¹⁻²³¹ in 20 mM sodium acetate-d₃, 2 mM sodium azide, (10% D₂O(v/v)) pH 5.55; β -form: 0.75 mM human PrP⁹¹⁻²³¹ in 20 mM sodium acetate-d₃, 2 mM sodium azide, (10% D₂O (v/v)) pH 4. 1D ¹H NMR

spectra were acquired with an acquisition time of 656 ms; ^1H , ^{15}N HSQC spectra with acquisition times of 328 ms and 168 ms in the direct and indirect dimensions respectively. NMR data were processed using Felix 97 (Molecular Simulations Inc). Proton chemical shifts were referenced 5 indirectly to TSP via the water signal.

5. *Aggregation of β -PrP observed by right angle light scattering*

Either oxidised human PrP pH 8.0 was diluted to 1 mg/ml in 2 mls of 10 mM NaAcetate + 10 mM Tris.HCl pH 8.0, or reduced human PrP pH 4.0 was diluted to 1 mg/ml in 2 mls of the same buffer at pH 4.0. The presence of aggregated material was monitored by right angle light scattering in a Schimadzu RF-5301 PC spectrofluorimeter with both excitation and emission monochromators set to slit width of 3 nm. 30 μl aliquots of 6M GuHCl were added and the solution allowed to equilibrate 15 for a few minutes before each reading was taken. All data were collected at 25°C.

6. *Electron Microscopy*

Reduced protein refolded at pH 4.0 to form β -sheet structure was examined using electron microscopy (EM). The specimens were prepared using standard negative stain procedures. Three microlitres of protein solution at a concentration of 0.25 mg/ml were pipetted onto carbon films 20 mounted on copper EM grids. After one minute the grids were washed with 80 microlitres of aqueous 2% uranyl acetate. The stain was left for approximately 10 sec before being blotted with filter paper. The grids were then inserted into a JEOL 1200 transmission electron microscope. Electron micrographs at approximately 1 micron underfocus were 25

recorded on Kodak SO-163 film under normal exposure conditions at 40,000 x magnification (calibrated against a grating) at 120 KeV. The defocus of the negatives was confirmed by optical diffractometry.

5

7. *Digestion with proteinase K*

Both α -PrP and β -PrP as a monomer and aggregate were subjected to digestion with varying concentrations of proteinase K (BDH) at 37°C for
10 1 hr. Protein was digested at a concentration of 1m/ml in 10mM NaAcetate + 10mM Tris. Acetate pH 8.0. Digestion was terminated by the addition of Pefablock (Boehringer Mannheim Corp.) to a final concentration of 1mM. Following the adition of Pefabloc samples were heated to 100°C for 5 mins in the presence of SDS loading buffer.
15 Aliquots of 20 μ l were subjected to SDS-PAGE and the gels stained with Coomassie brilliant blue.

Here we demonstrate the reversible interconversion of recombinant human
20 PrP between the native α -form, characteristic of PrP^c, and a similarly compact, highly soluble, monomeric form rich in β -structure which is stable in aqueous solution. Such an interconversion of a protein chain between two, discrete, monomeric backbone topologies is unprecedented. We further show that this soluble β -form (β -PrP) is a direct precursor of
25 fibrillar structures that are closely similar to those isolated from diseased brains. The conversion of PrP^c to β -PrP in suitable cellular compartments, and its subsequent stabilisation by intermolecular

associated, provides a possible molecular mechanism for prion propagation.

Human PrP⁹¹⁻²³¹ was expressed to high levels in *E. coli* as a protein aggregate and solubilised by extraction with 6 M guanidinium chloride and reducing agent. Subsequent purification, removal of denaturant and oxidation yielded a highly soluble, monomeric protein with a single intact disulphide bridge. Analysis of this refolded material by circular dichroism (CD) spectropolarimetry revealed a structure rich in a α -helical content (47%) with little β -sheet (18%) (Fig 1a legend). One-dimensional ¹H nuclear magnetic resonance (NMR) spectra (Fig 1b) and two-dimensional ¹H-¹⁵N correlation NMR spectra (data not shown) of this material show it to be conformationally similar to the previously determined mouse and hamster prion proteins^{3,4}, and a previously characterised human PrP⁹¹⁻²³¹ construct⁵.

In common with mouse PrP⁶, human PrP⁹¹⁻²³¹ folds and unfolds through a freely reversible transition ($\Delta G = -5.6$ Kcal./mol) between the fully native state and a random coil, with no detectable equilibrium intermediates. However, reduction of the disulphide bond in human PrP⁹¹⁻²³¹, and lowering the pH to 4.0 in a dilute acetate buffer in the absence of additives, generates a highly soluble protein which can be concentrated to at least 12 mg/ml. When the reduced protein is subjected to gel filtration, it elutes as a monomeric species (Fig 2). The CD signal in the amide region of the spectrum (Fig 1a) shows that this highly soluble reduced species adopts a radically different conformation from PrP^c. While the native state is characterised by a strong α -helical signal, the reduced form shows the shift to a conformation dominated by β -sheet. This constitutes

the first observation of a soluble monomeric β -form of the prion protein which opens up the opportunity for biophysical study.

This type of secondary structural transition has been well-documented in 5 proteins that undergo a switch from a soluble monomeric state to an aggregated fibrous and/or amyloid form in which β -structure is stabilised by inter-molecular interactions⁷. However, it is unprecedented for a protein to undergo such a β -sheet conversion while remaining in a monomeric state at high protein concentrations and in the absence of 10 denaturants. This is in contrast to the β -intermediate of mouse PrP¹²¹⁻¹²³⁸ which required the presence of denaturant for stabilisation. A similar folding intermediate of human α -PrP⁹¹⁻²³¹ exists but is poorly soluble. Clarified material has an increased apparent molecular weight of 40 kDa (Fig 2), indicative of tertiary disorder and expanded molecular volume. 15 Using the amide CD signal alone, it is uncertain whether the non-native compact conformation of human β -PrP⁹¹⁻²³¹ is sufficiently condensed to have immobilised side-chains characteristic of the native state of orthodox, globular proteins. However, the aromatic region of CD spectra contains signals from aromatic side-chains in asymmetric environments. Compared 20 to the native, oxidised molecule, the β -form retains a signal from aromatic residues but the intensity is diminished (Fig 1a). This result indicates that packed tertiary interactions present in PrP^c have been weakened, but not lost, in the β -conformation. Similarly, gel filtration of the reduced state reveals that it has, within the resolution of the technique, the same level of 25 compactness as the PrP^c conformation (Fig 2).

From the above measurement it is not clear whether the reduced form of the protein is classifiable as a molten globule or whether it is better

described as an alternative, fully folded conformation with well-defined tertiary interactions between side-chains. The term ‘molten globule’ was first used to describe distinct states adopted by some protein molecules when exposed to mildly denaturing conditions such as moderate concentrations of chaotropic agents (urea or guanidinium chloride) or acidic pH⁹. The chief signatures of the molten globule state are a well organised pattern of native-like backbone (secondary) structure with disordered side-chains and poorly defined tertiary interactions¹⁰. Originally, they were defined as equilibrium states but as more information became available on the behaviour of transiently populated, kinetic intermediates in folding reactions, often referred to as ‘I-states’ the definition has become blurred. This uncertainty is explained by the fact that I-states and molten globules have the above features in common, except that the former, kinetic intermediates are populated in native conditions. Despite this distinction, it has been shown for a number of proteins that molten globule states and I-states are experimentally indistinguishable¹¹. Moreover, because the I-state can be considered to be the denatured conformation in physiological conditions, it has attracted much attention with the context of cellular processes such as chaperone-assisted folding, protein transport between cellular compartments and amyloidosis.

Due to exposure of normally buried non-polar residues, it is rare for non-native states to show high solubility in the absence of denaturants. However, the availability of the β-form of PrP as a monomeric species at a concentration of 0.75 mM provided the opportunity of examining its physical properties using NMR. While the 1D ¹H-NMR spectrum of native human PrP⁹¹⁻²³¹ exhibits wide chemical shift dispersion characteristic of a fully folded globular protein, the spectrum of the β-

form of PrP exhibits considerably less chemical shift dispersion. This lack of dispersion is characteristic of the loss of fixed side chain interactions, a defining feature of molten globule states¹²⁻¹⁴. However, residual dispersion appears to be greater than that expected for a fully

5 unfolded protein (Fig 1b), implying some degree of tertiary packing in the β -form. This finding is consistent with the reduced but significant CD signal for the β -form in the aromatic region of the spectrum (Fig 1a). Therefore coupled with the amide CD data (Fig 1b), the NMR chemical shift data points to the β -form being predominantly molten globular in

10 nature. In addition, proton line-widths of the β -form are comparable to those observed in the native PrP^c conformation indicating that it is monomeric at the extremely high concentrations required for NMR and confirming the gel-filtration results.

15 The switch from α -to- β conformation is reversible. When the reduced β -form is exposed to a higher pH (8.0), the native α -conformation is restored. However, the rates of inter-conversion, in either direction, are extremely slow, requiring a period of days for completion (data not shown). This high kinetic barrier, however, can be side-stepped by fully

20 denaturing and refolding at the appropriate pH to generate either isoform.

By “fully denaturing” we include the meaning that there is no detectable secondary or tertiary structure ie the protein forms a “random coil”. Such denaturation can be determined by Circular Dichroism and/or NMR spectroscopy as described herein and can be achieved, for example, by maintaining the prion protein in 100mM DTT in 6M GuHCl + 10mM NaAcetate + 10mM NaAcetate + 10mM Tris. HCl pH 8.0 for 16 hours.

Solubility of the two isoforms is not equivalent. The α -form of PrP can be titrated with the denaturant guanidine hydrochloride (GuHCl) in order to determine equilibrium parameters for the folding pathway (data not shown). However, while the β -form of PrP is also highly soluble in aqueous buffers, titration with GuHCl leads to inter-molecular associations resulting in a visible precipitate (Fig 3). This material, when examined at high magnification, is initially composed of irregular spherical particles (Fig 4a) which associate over several hours to form fibrils (Fig 4b), very similar in appearance to those identified in diseased tissue.

10

PrP^{Sc} is characterised by its partial resistance to digestion with proteinase K (PK). As with native PrP^{C} , $\alpha\text{-PrP}$ is extremely sensitive to digestion with PK (Fig 5). However, $\beta\text{-PrP}$ shows marked protease resistance. This PK resistance is a function of the structural re-organisation of the monomeric β -form, with only a moderate further increase associated with aggregation (Fig 5). The different patterns of proteolytic cleavage fragments seen on PK digestion of $\alpha\text{-PrP}$ and $\beta\text{-PrP}$ provide further evidence of a major conformational re-arrangement in $\beta\text{-PrP}$. In marked contrast, the partially structured β -sheet conformation of reduced hamster PrP^{90-231} reported by Mehlhorn et al¹⁸ and Zhang et al (1997) Biochem, 36:12, 3542-3553¹⁹ is fully sensitive to PK digestion.

Unusually for a protein with a predominantly helical fold, the majority of residues in PrP^{91-231} have a preference for β -conformation (55% of non-glycine/proline residues). In view of this property, it is possible that the PrP molecule is delicately balanced between radically different folds with a high energy barrier between them; one dictated by local structural propensity (the β -conformation) and one requiring the precise docking of

side-chains (the native α -conformation). Such a balance would be influenced by mutations causing inherited human prion diseases¹⁵. It is also worthy of note that individuals homozygous for valine at polymorphic 129 of human PrP (where either methionine or valine can be encoded) are 5 more susceptible to iatrogenic CJD¹⁶, and valine has a much higher β -propensity than does methionine. Our results lend support to such a hypothesis by showing that the molecule is capable of slow interconversion between a native α and a non-native β conformation. Furthermore, we demonstrate that the β -form can be locked by 10 intermolecular association, thus supplying a plausible mechanism of propagation of a rare conformational state. It is possible that the PrP^c to β -PrP conversion we describe here, caused by reduction and mild acidification, is relevant to the conditions that PrP^c would encounter within the cell, following its internalisation during re-cycling. Such a mechanism 15 could underlie prion propagation, and account for the transmitted, sporadic and inherited aetiologies of prion disease. Initiation of a pathogenic self-propagating conversion reaction, with accumulation of aggregated β -PrP, may be induced by exposure to a 'seed' of aggregated β -PrP following prion inoculation, or as a rare stochastic conformational 20 change, or as an inevitable consequence of expression of a pathogenic PrP^c mutant which is predisposed to form β -PrP.

8. *Antibody production method*

25 Methods for purification of antigens and antibodies are described in Scopes, R.K. (1993) *Protein purification* 3rd Edition. Publisher - Springer Verlag. ISBN 0-387-94072-3 and 3-540-94072-3. The disclosure

of that reference, especially chapters 7 and 9, is incorporated herein by reference.

Antibodies may be produced in a number of ways.

5

- 1 The aberrant form of the prion protein eg β -form or aggregated thereof, especially a non-fibrillar aggregate, is purified from the same species as the immunization animal but will usually be human. The aberrant form may alternatively be prepared by purifying (from the animal or from a transferred host cell) the non-aberrant form and converting it to the aberrant form. The immunisation animal may be a "knock-out" mouse, with no prion protein at all. For monoclonal antibodies the animal is normally a mouse; for polyclonal, a rabbit or goat.
- 15 2. Raise antibodies to the antigen. For polyclonal antibodies, this is simply a matter of injecting suitably prepared sample into the animal at intervals, and testing its serum for the presence of antibodies (for details, see Dunbar, B.S. & Schwoebel, E.D. (1990) Preparation of polyclonal antibodies. *Methods Enzymol.* 182, 663-670). But it is essential that the antigen (ie. the protein of interest) be as pure as possible. For monoclonal antibodies, the purity of the antigen is relatively unimportant if the screening procedure to detect suitable clones uses a bioassay.
- 25 Antibodies can also be produced by molecular biology techniques, with expression in bacterial or other heterologous host cells (Chiswell, D.J. & McCafferty, J. (1992) Phage antibodies: will new "coli-clonal" antibodies replace monoclonal antibodies?" *Trends Biotechnol.* 10, 80-84). The purification method to be adopted will depend on the source material

(serum, cell culture, bacterial expression culture, etc.) and the purpose of the purification (research, diagnostic investigation, commercial production). The major methods are as follows:

- 5 1. *Ammonium sulphate precipitation.* The γ -globulins precipitate at a lower concentration than most other proteins, and a concentration of 33% saturation is sufficient. Either dissolve in 200g ammonium sulphate per litre of serum, or add 0.5 vol of saturated ammonium sulphate. Stir for 30 minutes, then collect the γ -globulin fraction by centrifugation, redissolve in an appropriate buffer, and remove excess ammonium sulphate by dialysis or gel filtration.
10
- 15 2. *Polyethylene glycol precipitation.* The low solubility of γ -globulins can also be exploited using PEG. Add 0.1 vol of a 50% solution of PEG 6,000 to the serum, stir for 30 minutes and collect the γ -globulins by centrifugation. Redissolve the precipitate in an appropriate buffer, and remove excess PEG by gel filtration on a column that fractionates in a range with a minimum around 6,000 Da.
20
- 25 3. *Isoelectric precipitation.* This is particularly suited for IgM molecules, and the precise conditions will depend on the exact properties of the antibody being produced.
4. *Ion-exchange chromatography.* Whereas most serum proteins have low isoelectric points, γ -globulins are isoelectric around neutrality, depending on the exact properties of the antibody being produced. Adsorption to cation exchangers in a buffer of around pH 6 has been

used successfully, with elution with a salt gradient, or even standard saline solution to allow immediate therapeutic use.

5. *Hydrophobic chromatography.* The low solubility of γ -globulins reflects their relatively hydrophobic character. In the presence of sodium or ammonium sulphate, they bind to many hydrophobic adsorbents, such as "T-gel" which consists of β -mercaptoethanol coupled to divinyl sulphone-activated agarose.

10 6. *Affinity adsorbents.* *Staphylococcus aureus* Outer coat protein, known as Protein A, is isolated from the bacterial cells, and it interacts very specifically and strongly with the invariant region (F_c) of immunoglobulins (Kessler, S.W. (1975) *Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: Parameters of the interaction of antibody-antigen complexes with protein A.* *J Immunol.* 115, 1617-1624. Protein A has been cloned, and is available in many different forms, but the most useful is as an affinity column: Protein A coupled to agarose. A mixture containing immunoglobulins is passed through the column, and only the immunoglobulins adsorb. Elution is carried out by lowering the pH; different types of IgG elute at different pHs, and so some trials will be needed each time. The differences in the immunoglobulins in this case are not due so much to the antibody specificity, but due to different types of F_c region. Each animal species produces several forms of heavy chain varying in the F_c region; for instance, mouse immunoglobulins include subclasses IgG₁, IgG_{2a}, and IgG₃ all of which behave differently on elution from Protein A.

Some γ -globulins do not bind well to Protein A. An alternative, Protein G from G from a *Streptococcus* sp., can be used. This is more satisfactory with immunoglobulins from farm animals such as sheep, goats and cattle, as well as with certain subclasses of mouse and rabbit IgGs.

5

The most specific affinity adsorbent is the antigen itself. The process of purifying an antibody on an antigen adsorbent is essentially the same as purifying the antigen on an antibody adsorbent. The antigen is coupled to the activated matrix, and the antibody-containing sample applied. Elution 10 requires a process for weakening the antibody-antigen complex. This is particularly useful for purifying a specific antibody from a polyclonal mixture.

Monoclonal antibodies (MAbs) can be prepared to most antigens. The 15 antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in 'Monoclonal Antibodies: A manual of techniques', H Zola (CRC Press, 20 1988) and in 'Monoclonal Hybridoma Antibodies: Techniques and Applications', J G R Hurrell (CRC Press, 1982).{PRIVATE }

Chimaeric antibodies are discussed by Neuberger *et al* (1988, 8th International Biotechnology Symposium Part 2, 792-799).

25

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to 5 constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parental antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is 10 independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner 15 domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites 20 is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

25 The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed

in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we 5 mean that the said antibodies and F(ab')₂ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

A CDR-grafted antibody may be produced having at least one chain wherein 10 the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from a second antibody (donor), the CDR-grafted antibody being capable of binding to the β -form PrP antigen.

15 The CDR-grafted chain may have two or all three CDRs derived from the donor antibody.

Advantageously, in the CDR-grafted chain, the or each CDR comprises a 20 composite CDR comprising all the residues from the CDR and all the residues in the corresponding hypervariable region of the donor antibody.

Preferably, at least one residue in the framework regions of the CDR-grafted chain has been altered so that it corresponds to the equivalent residue in the antibody, and the framework regions of the CDR-grafted 25 chain are derived from a human antibody.

Advantageously, the framework regions of the CDR-grafted chain are derived from a human Ig heavy chain. For such heavy chains, it is

preferred that residue 35 in the heavy chain framework regions be altered so that it corresponds to the equivalent residue in the donor antibody.

Suitably, for such heavy chains, at least one composite CDR comprising 5 residues 26 to 35, 50 to 65 or 95 to 102 respectively is grafted onto the human framework. It will be appreciated in this case that residue 35 will already correspond to the equivalent residue in the donor antibody.

Preferably, residues 23, 24 and 49 in such heavy chains correspond to the 10 equivalent residues in the antibody. It is more preferred that residues 6, 23, 24, 48 and 49 in such heavy chains correspond to the donor antibody in equivalent residue positions. If desired, residues 71, 73 and 79 can also so correspond.

15 To further optimise affinity, any one or any combination of residues 57, 58, 60, 88 and 91 may correspond to the equivalent residue in the donor antibody.

The heavy chain may be derived from the human KOL heavy chain. 20 However, it may also be derived from the human NEWM or EU heavy chain.

Alternatively, the framework regions of the CDR-grafted chain may be derived from a human kappa or lambda light chain. For such a light chain, 25 advantageously at least one composite CDR comprising residues 24 to 34, 50 to 56 or 89 to 97 respectively is grafted onto the human framework. Preferably, residue 49 also corresponds to the equivalent residue in the donor antibody.

To further optimise affinity, it is preferable to ensure that residues 49 and 89 correspond to the equivalent residues in the donor antibody. It may also be desirable to select equivalent donor residues that form salt bridges.

5 The light chain is preferably derived from the human REI light chain. However, it may also be derived from the human EU light chain.

Preferably, the CDR-grafted antibody comprises a light chain and a heavy chain, one or, preferably, both of which have been CDR-grafted in
10 accordance with the principles set out above for the individual light and heavy chains.

It is advantageous that all three CDRs on the heavy chain are altered and that minimal alteration is made to the light chain. It may be possible to alter
15 none, one or two of the light chain CDRs and still retain binding affinity at a reasonable level.

It will be appreciated that in some cases, for both heavy and light chains, the donor and acceptor residues may be identical at a particular position and
20 thus no change of acceptor framework residue will be required.

It will also be appreciated that in order to retain as far as possible the human nature of the CDR-grafted antibody, as few residue changes as possible should be made. It is envisaged that in many cases, it will not be necessary
25 to change more than the CDRs and a small number of framework residues. Only in exceptional cases will it be necessary to change a larger number of framework residues.

Preferably, the CDR-grafted antibody is a complete Ig, for example of isotype IgG₁, or IgG₂, IgG₃ or IgM.

If desired, one or more residues in the constant domains of the Ig may be
5 altered in order to alter the effector functions of the constant domains.

Preferably, the CDR-grafted antibody has an affinity for the β -form PrP antigen of between about $10^5 \cdot M^{-1}$ to about $10^{12} \cdot M^{-1}$, more preferably at least $10^8 \cdot M^{-1}$.

10

Advantageously, the or each CDR is derived from a mammalian antibody and preferably is derived from a murine MAb.

Suitably, the CDR-grafted antibody is produced by use of recombinant
15 DNA technology.

A further method for producing a CDR-grafted antibody comprises providing a first DNA sequence, encoding a first antibody chain in which the framework regions are predominantly derived from a first antibody
20 (acceptor) and at least one CDR is derived from a second antibody (acceptor), under the control of suitable upstream and downstream elements; transforming a host cell with the first DNA sequence; and culturing the transformed host cell so that a CDR-grafted antibody is produced.

25 Preferably, the method further comprises: providing a second DNA sequence, encoding a second antibody chain complementary to the first chain, under the control of suitable upstream and downstream elements; and transforming the host cell with both the first and second DNA sequences.

Advantageously, the second DNA sequence encodes a second antibody chain in which the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from the second antibody (donor).

5

The first and second DNA sequences may be present on the same vector. In this case, the sequences may be under the control of the same or different upstream and/or downstream elements.

10 Alternatively, the first and second DNA sequences may be present on different vectors.

A nucleotide sequence may be formed which encodes an antibody chain in which the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from a second antibody (donor), the antibody chain being capable of forming a CDR-grafted antibody.

15 The CDR-grafted antibodies may be produced by a variety of techniques, with expression in transfected cells, such as yeast, insect, CHO or myeloma cells, being preferred. Most preferably, the host cell is a CHO host cell.

To design a CDR-grafted antibody, it is first necessary to ascertain the variable domain sequence of an antibody having the desired binding properties. Suitable source cells for such DNA sequences include avian, mammalian or other vertebrate sources such as chickens, mice, rats and rabbits, and preferably mice. The variable domain sequences (V_H and V_L) may be determined from heavy and light chain cDNA, synthesized from the respective mRNA by techniques generally known to the art. The

hypervariable regions may then be determined using the Kabat method (Wu and Kabat, J. (1970) *J. Exp. Med.* **132**, 211). The CDRs may be determined by structural analysis using X-ray crystallography or molecular modelling techniques. A composite CDR may then be defined as containing
5 all the residues in one CDR and all the residues in the corresponding hypervariable region. These composite CDRs along with certain select residues from the framework region are preferably transferred as the "antigen binding sites", while the remainder of the antibody, such as the heavy and light chain constant domains and remaining framework regions,
10 may be based on human antibodies of different classes. Constant domains may be selected to have desired effector functions appropriate to the intended use of the antibody so constructed. For example, human IgG isotypes, IgG₁ and IgG₃, are effective for complement fixation and cell mediated lysis. For other purposes other isotypes, such as IgG₂ and IgG₄,
15 or other classes, such as IgM and IgE, may be more suitable.

For human therapy, it is particularly desirable to use human isotypes, to minimise antiglobulin responses during therapy. Human constant domain DNA sequences, preferably in conjunction with their variable domain
20 framework bases can be prepared in accordance with well-known procedures. An example of this is CAMPATH 1H available from Glaxo Wellcome.

Certain CDR-grafted antibodies are provided which contain select
25 alterations to the human-like framework region (in other words, outside of the CDRs of the variable domains), resulting in a CDR-grafted antibody with satisfactory binding affinity. Such binding affinity is preferably from about 10⁵.M⁻¹ to about 10¹².M⁻¹ and is more preferably at least about 10⁸.M⁻¹.

In constructing the CDR-grafted antibodies, the V_H and/or V_L gene segments may be altered by mutagenesis. One skilled in the art will also understand that various other nucleotides coding for amino acid residues or sequences contained in the Fc portion or other areas of the antibody may be altered in like manner (see, for example, PCT/US89/00297).

Exemplary techniques include the addition, deletion or nonconservative substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides, provided that the proper reading frame is maintained.

Substitutions, deletions, insertions or any subcombination may be used to arrive at a final construct. Since there are 64 possible codon sequences but only twenty known amino acids, the genetic code is degenerate in the sense that different codons may yield the same amino acid. Thus there is at least one codon for each amino acid, ie each codon yields a single amino acid and no other. It will be apparent that during translation, the proper reading frame must be maintained in order to obtain the proper amino acid sequence in the polypeptide ultimately produced.

Techniques for additions, deletions or substitutions at predetermined amino acid sites having a known sequence are well known. Exemplary techniques include oligonucleotide-mediated site-directed mutagenesis and the polymerase chain reaction.

Oligonucleotide site-directed mutagenesis in essence involves hybridizing an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template

for extension of the oligonucleotide to produce a strand containing the mutation. This technique, in various forms, is described in Zoller and Smith (1982) *Nucl. Acids Res.* **10**, 6487.

5 Polymerase chain reaction (PCR) in essence involves exponentially amplifying DNA *in vitro* using sequence specific oligonucleotides. The oligonucleotides can incorporate sequence alterations if desired. The polymerase chain reaction technique is described in Mullis and Fulloona (1987) *Meth. Enz.* **155**, 335. Examples of mutagenesis using PCR are
10 described in Ho *et al* (1989) *Gene* **77**, 51.

The nucleotide sequences, capable of ultimately expressing the desired CDR-grafted antibodies, can be formed from a variety of different polynucleotides (genomic DNA, cDNA, RNA or synthetic
15 oligonucleotides). At present, it is preferred that the polynucleotide sequence comprises a fusion of cDNA and genomic DNA. The polynucleotide sequence may encode various Ig components (eg V, J, D, and C domains). They may be constructed by a variety of different techniques. Joining appropriate genomic and cDNA sequences is presently
20 the most common method of production, but cDNA sequences may also be utilized (see EP-A-0 239 400).

9. *Raising an antibody response in a patient*

25 Active immunisation of the patient is preferred. In this approach, one or more β -form PrP proteins or an aggregate thereof, especially a non-fibrillar aggregate, are prepared in an immunogenic formulation containing suitable adjuvants and carriers and administered to the patient. Suitable adjuvants include Freund's complete or incomplete adjuvant, muramyl dipeptide, the

"Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminium hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic polyols or the Ribi adjuvant system (see, for example GB-A-2 189 141). "Pluronic" is a
5 Registered Trade Mark.

It may be advantageous to use a β -form PrP protein or an aggregate thereof from a species other than the one being treated, in order to provide for a greater immunogenic effect, although on the other hand maturing the
10 species may reduce the likelihood of creating anti- α PrP antibodies. Another compound can be used instead of the whole β -form PrP protein in order to produce inhibitory antibodies in the patient. Such other compounds may include fragments and analogues of the β -form PrP protein.

15 Skilled persons will appreciate that purification of the β -form and/or β -form binding agents, especially antibodies, can be accomplished by conventional techniques such as affinity chromatography or phage display. By " β -form binding agent" we include any agent which is able to binds preferentially the β -form rather than the α -form of a prion protein.
20 Purification of β -form aggregate binding agents, especially non-fibrillar aggregate binding agents, can also be accomplished by conventional techniques.

25 The binding agent is preferably an antibody or antigen binding fragment thereof such a Fab, Fv, ScFv and Ab, but it may also be any other ligand which exhibits the preferential binding characteristic mentioned above.

Affinity chromatography is described in Scopes, R. K. (1993) *Protein Purification: principles and practice* 3rd Ed. Springer-Verlag, New York, ISBN 0-387-44072-3, 3-540-94072-3. (See chapters 7 and 9 in particular).

5

Further information on the above affinity chromatography techniques and the immunoassay of antigen and antibody is provided by Roitt (1991) *Essential Immunology* 7th Ed. Blackwell Scientific Publications, London, ISBN 0-632-02877-7 (see chapter 5 in particular).

10

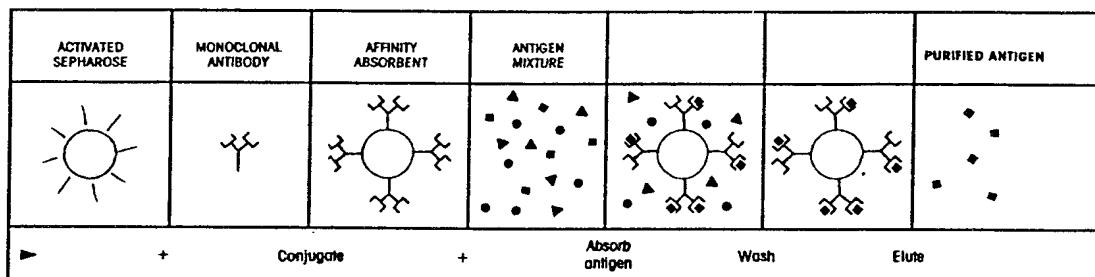
The disclosure of the above references is incorporated herein by reference. Nevertheless, an outline of known methods is described herein.

Purification of antigens and antibodies by affinity chromatography

15

Antigen or antibody is bound through its free amino groups to cyanogen-bromide-activated Sepharose particles. Insolubilized antibody, for example, can be used to pull the corresponding antigen out of solution in which it is present as one component of a complex mixture, by absorption to its surface. The unwanted material is washed away and the required ligand released from the affinity absorbent by disruption of the antigen-antibody bonds by changing the pH or adding chaotropic ions such as thiocyanate. Likewise, an antigen immunosorbent can be used to absorb out an antibody from a mixture whence it can be purified by elution. The potentially damaging effect of the eluting agent can be avoided by running the anti-serum down an affinity column so prepared as to have relatively weak binding for the antibody being purified; under these circumstances, the antibody is retarded in flow rate rather than being firmly bound. If a protein mixture is separated by iso-electric focusing

into discrete bands, an individual band can be used to affinity purify specific antibodies from a polyclonal antiserum.



5

Affinity chromatography. A column is filled with Sepharose-linked antibody. The antigen mixture is poured down the column. Only the antigen binds and is released by change in pH for example. An antigen-linked affinity column will purify antibody obviously.

Immunoassay of antigen and antibody with labelled reagents

10 Antigen and antibody can be used for the detection of each other and a variety of immunoassay techniques have been developed in which the final read-out of the reaction involves a reagent conjugated with an appropriate label. Radiolabelling with ^{131}I , ^{125}I , is an established technique.

Soluble Phase immunoassays

15

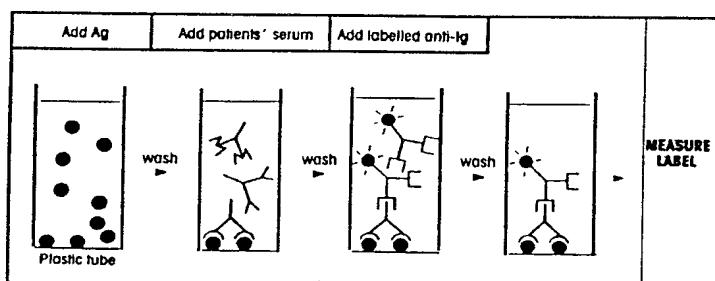
radioimmunoassay (RIA) for antigen

20 The binding of radioactively labelled antigen to a limited fixed amount of antibody can be partially inhibited by addition of unlabelled antigen and the extent of this inhibition can be used as a measure of the unlabelled material added.

For antibody

The antibody content of a serum can be assessed by the ability to bind to antigen which has been in and immobilised by physical absorption to a plastic tube or micro-agglutination tray with multiple wells; the bound immunoglobulin may then be estimated by addition of a labelled anti-Ig raised for another species. For example, a patient's serum is added to a microwell coated with antigen, the antibodies will bind to the plastic and remaining serum proteins can be readily washed away. Bound antibody can be estimated by addition of ^{125}I -labelled purified rabbit anti IgG; after rinsing out excess unbound reagent, the radioactivity of the tube will be a measure of the antibody content of the patient's serum. The distribution of antibody in different classes can obviously be determined by using specific antisera.

15

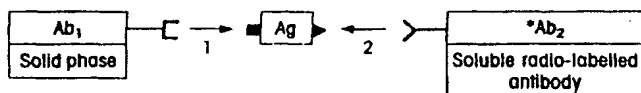


Solid phase immunoassay for antibody. By attaching antibody to the solid phase, the system can be used to assay antigen. To reduce non-specific binding of IgG to the solid phase after absorption of the first reagent, it is usual to add an irrelevant protein such as gelatin, or more recently α_1 - glycoprotein, to block any free sites on the plastic

Immunoradiometric assay for antigen

20 This differs from radioimmunoassay in the sense that the labelled reagent is used in excess. For the estimation of antigen, antibodies are coated on to a solid surface such as plastic and the test antigen solution added; after washing, the amount of antigen bound to the plastic can be estimated by adding an excess of radio-labelled antibody. The specificity of the method

can be improved by the sandwich assay which uses solid phase and labelled antibodies with specificities for different parts of the antigen:



Because of health hazards and the deterioration of reagents through
5 radiation damage, types of label other than radiosotopes have been sought.

ELISA (enzyme-linked immunosorbent assay)

Perhaps the most widespread alternative has been the use of enzymes
10 which give a coloured reaction product, usually in solid phase assays.
Enzymes such as horse radish peroxidase and phosphatase have been
widely employed. A way of amplifying the phosphatase reaction is to use
NADP as a substrate to generate NAD which now acts as a coenzyme for
a second enzyme system. Pyrophosphatase from *E.coli* provides a good
15 conjugate because the enzyme is not present in tissues, is stable and gives
a good reaction colour. Chemi-luminescent systems based on enzymes
such as luciferase can also be used.

Conjugation with the vitamin biotin is frequently used since this can
20 readily be detected by its reaction with enzyme-linked avidin or
streptavidin to which it binds with great specificity and affinity.

10. Identification of ligands by phage display

25 The display of proteins and polypeptides on the surface of bacteriophage
(phage), fused to one of the phage coat proteins, provides a powerful tool
for the selection of specific ligands. This 'phage display' technique was

originally used by Smith in 1985 (*Science* 228, 1315-7) to create large libraries of antibodies for the purpose of selecting those with high affinity for a particular antigen. More recently, the method has been employed to present peptides, domains of proteins and intact proteins at the surface of 5 phages in order to identify ligands having desired properties.

The principles behind phage display technology are as follows:

- (i) Nucleic acid encoding the protein or polypeptide for display is cloned 10 into a phage;
- (ii) The cloned nucleic acid is expressed fused to the coat-anchoring part of one of the phage coat proteins (typically the p3 or p8 coat proteins in the case of filamentous phage), such that the foreign protein or polypeptide is displayed on the surface of the phage;
- 15 (iii) The phage displaying the protein or polypeptide with the desired properties is then selected (*e.g.* by affinity chromatography) thereby providing a genotype (linked to a phenotype) that can be sequenced, multiplied and transferred to other expression systems.

- 20 Alternatively, the foreign protein or polypeptide may be expressed using a phagemid vector (*i.e.* a vector comprising origins of replication derived from a phage and a plasmid) that can be packaged as a single stranded nucleic acid in a bacteriophage coat. When phagemid vectors are employed, a “helper phage” is used to supply the functions of replication and packaging of the phagemid nucleic acid. The resulting phage will express both the wild type coat protein (encoded by the helper phage) and the modified coat protein (encoded by the phagemid), whereas only the modified coat protein is expressed when a phage vector is used.

Methods of selecting phage expressing a protein or peptide with a desired specificity are known in the art. For example, a widely used method is "panning", in which phage stocks displaying ligands are exposed to solid phase coupled target molecules, *e.g.* using affinity chromatography.

5

Alternative methods of selecting phage of interest include SAP (Selection and Amplification of Phages; as described in WO 95/16027) and SIP (Selectively-Infective Phage; EP 614989A, WO 99/07842), which employ selection based on the amplification of phages in which the displayed 10 ligand specifically binds to a ligand binder. In one embodiment of the SAP method, this is achieved by using non-infectious phage and connecting the ligand binder of interest to the N-terminal part of p3. Thus, if the ligand binder specifically binds to the displayed ligand, the otherwise non-infective ligand-expressing phage is provided with the parts 15 of p3 needed for infection. Since this interaction is reversible, selection can then be based on kinetic parameters (see Duenas *et al.*, 1996, *Mol. Immunol.* 33, 279-285).

The use of phage display to isolate ligands that bind biologically relevant 20 molecules has been reviewed in Felici *et al.* (1995) *Biotechnol. Annual Rev.* 1, 149-183, Katz (1997) *Annual Rev. Biophys. Biomol. Struct.* 26, 27-45 and Hoogenboom *et al.* (1998) *Immunotechnology* 4(1), 1-20. Several randomised combinatorial peptide libraries have been constructed 25 to select for polypeptides that bind different targets, *e.g.* cell surface receptors or DNA (reviewed by Kay, 1995, *Perspect. Drug Discovery Des.* 2, 251-268; Kay and Paul, 1996, *Mol. Divers.* 1, 139-140). Proteins and multimeric proteins have been successfully phage-displayed as functional molecules (see EP 0349578A, EP 0527839A, EP 0589877A; Chiswell and McCafferty, 1992, *Trends Biotechnol.* 10, 80-84). In

addition, functional antibody fragments (*e.g.* Fab, single chain Fv [scFv]) have been expressed (McCafferty *et al.*, 1990, *Nature* **348**, 552-554; Barbas *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**, 7978-7982; Clackson *et al.*, 1991, *Nature* **352**, 624-628), and some of the shortcomings of 5 human monoclonal antibody technology have been superseded since human high affinity antibody fragments have been isolated (Marks *et al.*, 1991, *J. Mol. Biol.* **222**, 581-597; Hoogenboom and Winter, 1992, *J. Mol. Biol.* **227**, 381-388). Further information on the principles and practice of phage display is provided in *Phage display of peptides and 10 proteins: a laboratory manual* Ed Kay, Winter and McCafferty (1996) Academic Press, Inc ISBN 0-12-402380-0, the disclosure of which is incorporated herein by reference.

11. Immunisation - Preferred protocols

15

11a. Preparation of antigen

For the preparation of monoclonal antibodies (mAbs), β -PrP or its derivatives may be provided in an acetate buffer as described 20 above. Antigens may be physically (by creating recombinant β -PrP fusion proteins) or chemically coupled to suitable carrier proteins to provide additional T cell help for immunisation in PRNP^{+/+} mice and other rodents.

25

11b. Mice of various strains, rats, hamsters or rabbits can be inoculated subcutaneously with β -PrP (or an aggregate thereof, especially a non-fibrillar aggregate (50-100 μ g/ animal), emulsified in complete/incomplete Freunds adjuvant at 3 weekly intervals (Days 0,20,41). At day 37 anti-peptide activity can be assayed by

ELISA. On day 48 in the case of animals used for mAb production, a final intraperitoneal boost can be given and the animals killed for fusion 3 days later (day 50). In the case of rabbits inoculated to produce polyclonal antibodies, the animals may be bled after the final boost, and at regular subsequent intervals with or without further inoculation depending on anti- β PrP titre.

12. *Monoclonal antibody preparation*

10

Routine methods may be used (Galfre G., and Milstein, C. 1981 *Methods in Enzymology* 73, 3-46)

12a. *Myeloma cells*

15

The following fusion partners may be used:

Mouse	NSO/u	Clark M.R., and Milstein, C. 1982 <i>Somatic Cells Genetics</i> 7, 657-666
	X63/Ag 8.653	Keraney <i>et al.</i> 1979 <i>J. Immunol.</i> 123, 1548-1550
	SP2/0	Sanchez-Madrid <i>et al</i> 1983 <i>J. Immunol.</i> 130, 309-312
20		Bluestone 1987 <i>PNAS</i> 84, 1374
25	Rat fusions	Y3 (210.RCY3.Ag 1.2.3)YO Galfre G., and Milstein, C. 1981 <i>Methods in Enzymology</i> 73, 3-46
30	Hamster fusions	SP2/0

11b. Fusion procedure

Two spleens from mice that have produced high titre antibody are fused. Myeloma cells growing in exponential phase may be mixed
5 with splenic single cell suspensions in appropriate ratios, washed free of serum, and then gently resuspended in a 50% polyethylene glycol solution at 37°C followed after 1-2 minutes with increasing volumes of serum-free medium. After a further incubation in RPMI/10% foetal calf serum (RF₁₀) at 37°C for 30 minutes, the hybridomas may be washed and resuspended in HAT medium and
10 hybridoma growth supplements, are cultured in 200 µl flat-bottomed tissue culture wells at 37°C in 5% CO₂ enriched humidified air. The cultures remain in RF₁₀/HAT medium for 2 weeks, and are then maintained in RF₁₀/HT medium for a further
15 week and thereafter in RF₁₀. At day 10-14 positive wells are screened for anti-PrP antibody by ELISA. Positive wells are then repeatedly cloned by limiting dilution until stable. Hybridomas cryopreserved in FCS 10% DMSO are stored in liquid N₂ dewars.

20 13. Screening for anti-β PrP antibodies in serum

Recombinant PrP (0.5-10µg/well), may be dialysed against appropriate coating buffer (pH 4-10) and adsorbed to standard ELISA plates for 30-60 minutes at 37°C prior to washing x4 in PBS/Tween 0.05% (PBST). After
25 blocking in PBS/BSA 2% with or without additional sera, dilutions of serum are incubated in duplicate as are relevant negative and positive controls. After washing, the peroxidase conjugated anti-IgG secondary is incubated, washed and then fresh ortho-phenyl diamine (OPD) substrate

added. Finally after stopping the reaction with 3M sulphuric acid the absorbance is measured at 492nm.

5 **14. Screening culture supernatants for PrP^{Sc}-specific monoclonal antibodies**

This may involve a staged two day procedure. On day 1, 50µl of the growing cultures may be screened for anti-β PrP IgG as in the ELISA described above. This β-PrP may or may not be first digested with 10 proteinase K to remove any alpha PrP species. Positive wells in this assay may then be screened the following day in a dot blot assay modified from Collinge et al 1995 *Lancet* 346:569-570. Dot blot apparatus (ELIFA, Pierce Wariner) can be used that allows the simultaneous screening of multiple supernatants. Supernatants can be screened for binding to 15 recombinant β-PrP, 1% normal human brain homogenate and to a pool of 1% homogenates from CJD brains containing types 1-4, thus enabling the preferential selection of PrP^{Sc}-specific mAbs. Thus only mAbs that bind infectious prions and not PrP^c from normal brain will be expanded. Alternatively, culture supernatants can be screened for preferential 20 binding to either alpha or β-PrP, or to synthetic peptides to which PrP^{Sc}-specific mAbs may bind. The 15B3 PrP^{Sc}-specific mAb cross-reacts with human, bovine and murine PrP^{Sc}, and its epitope has been mapped with linear synthetic peptides to three regions on the bovine PrP molecule: residues 142-148, 162-170 and 214-226 and later two of which may not be 25 recognised by antibodies that bind to both PrP^c and PrP^{Sc} (Korth C. et al. 1997 *Nature* 390, 74-77). These peptides are adsorbed to ELISA plates with poly-lysine.

Monoclonal antibodies raised against β-PrP

β-PrP is highly immunogenic in *Prn-p^{0/0}* (PrP null) mice immunised subcutaneously with soluble or aggregated protein emulsified in Freund's adjuvant and splenocytes from hyperimmunised PrP null mice can be readily fused with various fusion partners (eg NSO, NS1 murine myeloma cells). One of the major advances of using β-PrP when making monoclonal antibodies is its use in screening. Previously, high throughput hybridoma screening has not been possible given the small amounts of available purifiable native PrP. We have now developed a rapid PrP^c/PrP^{sc} discriminating ELISA screening protocol using recombinant PrP folded into either alpha or beta conformations. To date we have found that some mAbs recognise only alpha PrP and others recognise both alpha and beta conformations. We presume that PrP^{sc}-specific mAbs will recognise recombinant beta PrP and not alpha recombinant protein. An early rejection of alpha-only binding mAbs dramatically increases the efficiency of the screening process. Additional information regarding mAb epitopes has been obtained using responses to recombinant alpha and beta PrP pre-digested or not with varying concentrations of proteinase K.

20

We have now produced 32 monoclonal anti-PrP antibodies using standard hybridoma technology. The majority of these mAbs recognise native alpha PrP in dot blots and on the surface of a wide variety of cells in flow cytometric analyses as well as denatured PrP derived from normal or TSE brain homogenates.

15. Characterisation of mAbs

Immunoglobulin subclass and culture supernatant Ig concentration can be measured by standard ELISA techniques. The fine specificity of PrP^c or 5 PrP^{Sc} specific mAbs can be defined either by using a gridded array of overlapping human PrP peptides (synthesised commercially by Jerino Bio Tools GMbH) or by using pools of PrP synthetic peptides (synthesised individually using standard f-moc chemistry) in the standard ELISA. Measurements of the affinity of anti-PrP mAbs for their ligands can be 10 made using surface plasmon resonance. Direct comparisons can be made of mAb binding to alpha and β-PrP molecules.

16. Binding of mAbs to surface bound and intracellular PrP

15 Flow cytometry and immunofluorescence microscopy may be used to study surface and intracellular PrP^c/PrP^{Sc} expression in cell lines that express surface PrP (eg EVBV lymphoblastoid, U937, K562, HEI) and peripheral blood mononuclear cells.

20 17. Binding to PrP in tissue sections

Both acetone fixed fresh frozen sections and fixed paraffin embedded sections from normal and CJD/BSE/scrapie tissue can be used to assess the usefulness of β-PrP binding mAbs in routine immunohistochemistry.

25

18. Use of antibody in the diagnosis of a prion disease

The detection of the disease-associated isoform of prion protein, PrP^{Sc}, in brain or other tissues from patients is thought to be diagnostic of prion

disease. To distinguish PrP^{Sc} from its cellular precursor, PrP^c, requires either pre-treatment with proteinase K, which will completely digest PrP^c, but only removes a protease-sensitive N-terminal of PrP^{Sc} or, alternatively, would require an antibody which distinguished between 5 PrP^c and PrP^{Sc}. Only one such selective antibody (Korth C. *et al.* 1997 *Nature* 390, 74-77) has yet been reported and appears to be able to selectively immunoprecipitate PrP^{Sc}. It is not clear as yet, however, whether this antibody offers any increase in diagnostic sensitivity over existing monoclonals. It is an IgM antibody and is likely to be of low 10 affinity for PrP^{Sc}. By using recombinant human PrP, and in particular the β-form of the invention, or an aggregate thereof, especially a non-fibrillar aggregate, we should produce antibodies with high diagnostic sensitivity as well as specificity. Anti β-PrP antibodies may be PrP^{Sc}-specific or, alternatively, detect low levels of β-PrP monomer in blood or other tissues 15 or bodily fluids or materials, including faeces, urine, sputum, lymph, lymph nodes, tonsil, appendix tissue, cerebrospinal fluid, or derivatives or components thereof.

Skilled persons will appreciate that the β -form specific binding agents such as antibodies of the invention can be used in subtraction assays which involve pretreatment of a sample with a binding agent such as an antibody

5 specific for the normal cellular α -form of a prion protein, Prp^C, followed by treatment with a β -form specific binding agent eg antibody and detection of anti β -form binding. The pre-treatment step increases the sensitivity of the assay for the β -form.

10 Similar subtraction methods are described in WO98/16834.

Many detection systems are available for using a monoclonal antibody to diagnose a disease. A number of possibilities are discussed below:

15 **19. *Detection of PrP^{Sc} in body fluids or tissue homogenates***

a. Sandwich ELISA can be used to detect PrP^{Sc} in body fluids eg serum or cerebrospinal fluid (CSF). This relies on using immobilised ultrasensitive PrP^{Sc}-specific mAbs to capture PrP^{Sc} in solution and then using biotinylated mAbs or rabbit polyclonal antiserum with specificity for alternative PrP epitopes to detect the immobilised complexes. The same techniques can be used to detect PrP^{Sc} in tissue homogenates.

20

b. Dot blots may be used. Here tissue homogenates are placed directly on a suitable membrane and be treated with proteinase K to remove PrP^C. The membrane can be incubated with anti-PrP antibodies and then such binding detected using an appropriate,

labelled secondary antibody. Various labelling systems, involving enzymatic, fluorescent, radioisotopic or chemiluminescent methods are commonly used.

5 c. Standard Western blotting techniques can be used. These methods allow not only the detection of PrP, but of specific patterns of banding following proteinase K digestion. These patterns allow the recognition of distinct strains of prions and allow, for instance, the differentiation of new variant CJD from classical CJD (see
10 Collinge *et al.* 1996 *Nature* 383, 685-690 and international PCT patent application published as WO 98/16834).

15 d. Diagnostic methods may be developed based on the differential affinity of anti-PrP mAbs for PrP^c and PrP^{Sc}. Surface plasmon resonance is ideally suited for this purpose. In such assays, purified anti-PrP mAbs are immobilised and binding to solubilised PrP measured directly from tissue fluids and homogenates. Enrichment of PrP^{Sc} by differential centrifugation or affinity purification may be required prior to the above assays.
20

20. *Detection of cell associated PrP^{Sc}*

It is likely that the levels of PrP^{Sc} in peripheral blood mononuclear cells (PBMC) of vCJD patients will be low and detection will depend on
25 optimising methods for surface and intracellular detection of PrP and then identifying lymphocyte sub-populations with the highest prion load. Anti- β PrP mAbs can be purified and conjugated to biotin or fluorochromes for this purpose. Dual and three colour flow cytometry can be used to identify the PrP^{Sc} bearing cell types. After surface staining by

conventional techniques, intracellular PrP can be detected after fixation and permeabilisation of the cell membranes. Cellular manipulation (eg stimulation of proliferation of the pharmacological blockade of intracellular secretory or endocytic pathways) may be used to enhance PrP detection.

21. *Immunohistochemistry*

Prion disease may be diagnosed by abnormal patterns of PrP immunoreactivity on either formalin fixed, or frozen, tissue sections using established immunohistochemical detection techniques. Frozen tissue sections of whole brains (histoblots) may be treated with proteinase K and similarly exposed to antibodies to detect patterns of PrP^{Sc} deposition which may also allow discrimination of prion strain types.

15

22. *Detection of anti-PrP^{Sc} antibodies in TSE*

Although it is assumed that anti-PrP^{Sc} is not induced during the course of natural scrapie infection, this has not been studied systematically in any form of CJD. Thus to detect anti-PrP^{Sc} we may absorb β-PrP to immunosorbent plates and perform standard ELISA as above.

23. *Detection of PrP using highly sensitive in vitro lymphocyte assays*

25 Specific T cells are extremely sensitive to the presence of their cognate antigen. PrP-specific T cell lines/clones raised in PRNP^{0/0} mice can be used to detect PrP^{Sc} after its absorption to immunomagnetic particles using PrP^{Sc}-specific mAbs (after Hawke et al 1992 *Journal of Immunological Methods* 155(1):41-48). In this method PrP^{Sc} absorbed to the particles is

co-cultured with specific T lymphocytes and antigen presenting cells and proliferation (using standard ^3H -thymidine incorporation assays) and/or cytokine release is measured.

5 **24. *Toxicity of β -PrP***

To examine the effect of β -PrP, *in vivo*, mice were inoculated with soluble (low salt) and aggregated (200mM NaCl) forms of the recombinant murine protein. The recombinant, cellular PrP^C form was also included in the
10 experiment as a control.

By "low salt" we mean an ionic strength which is insufficient to cause aggregation of β -PrP, for example 0 mM to 25 mM.

15 The salt-treated, aggregated β -PrP material has two forms, as identified by electron microscopy. Addition of 200mM NaCl causes a rapid formation (<1hour) of spherical particles (10-20 nm diameter) and further incubation (>24 hours) leads to the formation of fibrillar structures.
20 Because salt addition leads to a time-dependent change in the structure of β -PrP, three different inocula were used: low salt, short salt incubation (2-5 minutes) and long salt incubation (30 hours).

In order to test whether any pathological effects were dependent on expression of PrP^C in the recipient, two mouse genotypes were used:
25 TG20 (over-expressing mouse PrP) and SV129/B6 (PrP ablated).

Ablated mice are described in Beuler, H., 1992 *Nature* 356:577-582.

TG20 mice are described in Fischer, M., 1996 *The EMBO Journal* 15(6):1255-1264.

5 Animals were anaesthetised and inoculated intra-cranially with 30 μ L aliquots of protein solution (1.6 mg/ml). After recovery from the anaesthetic some of the mice suffered immediate and severe fits and died within 5 minutes. This acute toxicity was most prevalent in the TG20 mice after inoculation with β -PrP which had undergone a short salt incubation. The PrP-ablated mice showed no susceptibility to β -PrP in
10 any of its 3 forms. The results are given in the table below.

	TG20 (PrP ^C over expression)	SV129/B6 (PrP ablated)
PrP ^C	0 / 8	N.D.
β -PrP – soluble, low salt	4 / 10	0 / 10
β -PrP – 200 mM NaCl short incubation	5 / 10	0 / 10
β - PrP – 200mM NaCl long incubation	1 / 10	1 / 10
Buffer control	0 / 10	N.D

N.D.=none detected.

15 The toxicity of β -PrP in these circumstances is acute and therefore it can be argued that the effect is unlike that seen in chronic T.S.E.s. However, the amount of PrP material introduced into the brain (~50 μ g) is extremely large and, more importantly, the effect is mediated by PrP^C. Given that

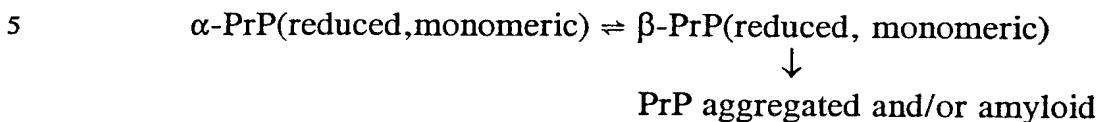
T.S.E.s can only infect animals which express PrP^C, it is likely that the effects elicited by β-PrP in this experiment are relevant to prion diseases. One hypothesis which is consistent with the above observations is that the toxic agent in T.S.E.s is not the fibrillar insoluble material but a
5 transiently formed low molecular weight form which goes on to form these high-order aggregates. This toxic material never reaches high steady-state levels during the disease and so the rate of synaptic loss and cell death is slow. When large quantities are introduced in a single dose then there is a sudden, widespread effect on neurones which, in this initial
10 phase, leads to sustained depolarisation and the consequent fits. The fact that this effect is only seen on neurones with endogenous PrP^C suggests that the effect is mediated by interactions between β-PrP and PrP^C. In the chronic Prion diseases there is only sufficient β-PrP at any one time to affect a small number of neurones, but long-term exposure to low levels of
15 the agent leads to a slow loss of synaptic connections and eventual death of cells. We term this lethal form of the protein β-PrP^L.

This represents the first occasion on which toxic Prions have been made *in vitro* and the results demonstrate the importance of our production and
20 characterisation of the soluble β-form precursor of the toxic aggregated material.

25. *Identification of compounds capable of inhibiting and/or reversing conversion of a prion protein from its α conformation to a β-conformation or from β-form to aggregated and/or amyloid form, especially a non-fibrillar aggregate.*

Use of β -PrP in high-throughput screening for potential therapeutics

The experiments thus far performed on the β -PrP structure can be summarised:



The first transition is reversible, with the β -PrP conformation being favoured by lowering the pH to an acidic pH, for example pH 4. The second transition is effectively irreversible and results in the formation of the aggregated and/or amyloid, especially a non-fibrillar aggregate, form which scatters light owing to the large particle size. The system can be kept in the monomeric β -PrP form by maintaining a low ionic strength eg 20mM NaCl or equivalent. When the ionic strength is raised (by use of guanidinium chloride, sodium chloride, or potassium chloride at a concentration of from 100-200 mM, especially 200 mM or more, for instance) the system shifts towards the aggregated and/or amyloid state.

The availability and understanding of this system allows the design of routine and rapid assays for compounds which prevent aggregated and/or amyloid formation, especially the toxic non-fibrillar aggregate mentioned in section 24.

The simplest and technically most direct method is to screen for any compound which blocks the second transition by poising the system in the β -PrP (reduced, monomeric) state at pH 4 and low ionic strength, for example 20mM NaCl. Compounds will then be added to this protein solution and incubated in screening wells. The next step will be to increase the ionic strength by the addition of NaCl, KCl or similar

compound which would normally promote the formation of the aggregated and/or amyloid form and cause an increase in light scattering in the 400-500nm range of wavelengths. Any compound, added at the first stage, which was capable of binding to and stabilising either the α -PrP (reduced, monomeric) form and/or the β -PrP (reduced, monomeric) form will show a low scattering signal in the relevant well.

Such a system can be rapidly optimised for a high throughput screen by use of large, multi-well microtitre plates handled by robotic systems. Screening of hundreds of thousands of different compounds is then entirely feasible over a timescale of several months. Even larger scale screens, of millions of compounds, is also entirely possible with allocation of sufficient technical resources. Assuming sufficient diversity within the chemical libraries screened, it ought to be possible to identify compounds which inhibit β -PrP or aggregated β -PrP formation at extremely low concentration, which can then be further evaluated.

Recombinant β -PrP: vaccine potential

Disruption of the transformation of normal cellular PrP is potentially achievable using antibodies directed at either PrP^c or PrP^{sc} or both. However, it has long been recognised that anti-PrP immunity is not induced during the course of natural TSE. This can be most readily explained by the widespread expression of tolerogenic levels of PrP in the lymphoreticular system; particularly in the thymus where T cells develop. Unless helper T cells are stimulated by an immunogen, B cells will not be driven to differentiate into antibody-secreting plasma cells. It is known that physical linkage of a 'carrier' protein to the antibody target may overcome the need for its recognition by T cells. Despite the fact that

PrP^c is expressed on many haemopoetic cells in the bone marrow making tolerance of PrP-binding B cells also likely, we have been able to conjugate carrier proteins to both recombinant alpha and beta PrP and induce anti-PrP antibodies in wild-type mice; even using mouse recombinant protein conjugates as immunogens. We have also found that T cell help can be provided by immunising mice with human recombinant PrP in either alpha or beta conformations. Presumably the sequence differences between mouse and human PrP are the stimulating T cell epitopes. Both of these approaches are currently being tested for disease modifying potential and they may form the basis of therapeutic/preventative vaccination for CJD and other TSE.

15 26. *Production of compounds comprising a portion capable of binding
preferentially to the β -form of a prion protein and a further
effector portion*

In one preferred embodiment the compound comprises an effector portion
20 which is directly or indirectly cytotoxic.

Methods for the preparation of compounds which possess a target-specific binding portion and a directly, or indirectly, cytotoxic portion are well known in the art.

25

For example, Bagshawe and his co-workers have disclosed (Bagshawe (1987) *Br. J. Cancer* 56, 531; Bagshawe *et al.* (1988) *Br. J. Cancer* 58, 700; WO 88/07378) conjugated compounds comprising an antibody or part thereof and an enzyme which converts an innocuous pro-drug into a

cytotoxic compound. The cytotoxic compounds were alkylating agents, e.g. a benzoic acid mustard released from para-N-bis(2-chloroethyl)aminobenzoyl glutamic acid by the action of *Pseudomonas sp.* CPG2 enzyme.

5

An alternative system using different pro-drugs has been disclosed (WO 91/11201) by Epenetos and co-workers. The cytotoxic compounds were cyanogenic monosaccharides or disaccharides, such as the plant compound amygdalin, which releases cyanide upon the action of a β -glucosidase and 10 hydroxynitrile lyase.

In a further alternative system, the use of antibody-enzyme conjugates containing the enzyme alkaline phosphatase in conjunction with the pro-drug etoposide 4'-phosphate or 7-(2'aminoethyl phosphate) mitomycin or a 15 combination thereof have been disclosed (EP 0 302 473; Senter et al., (1988) *Proc. Natl. Acad. Sci. USA* 85, 4842).

Another approach is the *in vivo* application of streptavidin conjugated antibodies followed, after an appropriate period, by radioactive biotin 20 (Hnatowich et al. (1988) *J. Nucl. Med.* 29, 1428-1434), or injection of a biotinylated mAb followed by radioactive streptavidin (Paganelli et al. (1990) *Int. J. Cancer* 45, 1184-1189).

Further examples of the targeting of compounds which are directly, or 25 indirectly, cytotoxic are disclosed in PCT/GB94/00087 (EP 0 815 872 A2).

27. Exemplary pharmaceutical formulations of the invention

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention β -form of a prion protein or an aggregate thereof, or a binding agent, including antibody) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

10

Whilst it is possible for an agent eg compound of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the agent of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

25

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg

povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable
5 machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

10

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and
15 mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with
20 the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid
25 carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

10 The following examples illustrate pharmaceutical formulations according to the invention in which the active ingredient is selected from one or more of antibodies and agents eg compounds of the invention:

Example A: Tablet

15

Active ingredient	100 mg
Lactose	200 mg
Starch	50 mg
Polyvinylpyrrolidone	5 mg
20 Magnesium stearate	4 mg
	359 mg

Tablets are prepared from the foregoing ingredients by wet granulation followed by compression.

Example B: Ophthalmic Solution

Active ingredient 0.5 g

Sodium chloride, analytical grade 0.9 g
Thiomersal 0.001 g
Purified water to 100 ml
pH adjusted to 7.5

5

Example C: Tablet Formulations

The following formulations A and B are prepared by wet granulation of the ingredients with a solution of povidone, followed by addition of magnesium stearate and compression.
10

Formulation A

		<u>mg/tablet</u>	<u>mg/tablet</u>
	(a) Active ingredient	250	250
15	(b) Lactose B.P.	210	26
	(c) Povidone B.P.	15	9
	(d) Sodium Starch Glycolate	20	12
	(e) Magnesium Stearate	5	3
		—	
20		500	300

Formulation B

		<u>mg/tablet</u>	<u>mg/tablet</u>
	(a) Active ingredient	250	250
	(b) Lactose	150	-
5	(c) Avicel PH 101°	60	26
	(d) Povidone B.P.	15	9
	(e) Sodium Starch Glycolate	20	12
	(f) Magnesium Stearate	5	3
		—	
10		500	300

Formulation C

	<u>mg/tablet</u>
	Active ingredient
15	100
	Lactose
	200
	Starch
	50
	Povidone
	5
	Magnesium stearate
	4
20	359

The following formulations, D and E, are prepared by direct compression of the admixed ingredients. The lactose used in formulation E is of the direction compression type.

Formulation D

	<u>mg/capsule</u>
Active Ingredient	250
Pregelatinised Starch NF15	150
	5
	400

Formulation E

	<u>mg/capsule</u>
10 Active Ingredient	250
Lactose	150
Avicel [®]	100
	15
	500

Formulation F (Controlled Release Formulation)

The formulation is prepared by wet granulation of the ingredients (below) with a solution of povidone followed by the addition of magnesium stearate 20 and compression.

	<u>mg/tablet</u>
(a) Active Ingredient	500
(b) Hydroxypropylmethylcellulose	112
	(Methocel K4M Premium) [®]
25 (c) Lactose B.P.	53
(d) Povidone B.P.C.	28
(e) Magnesium Stearate	7

700

Drug release takes place over a period of about 6-8 hours and is generally complete after 12 hours.

5 Example D: Capsule Formulations

Formulation A

A capsule formulation is prepared by admixing the ingredients of
10 Formulation D in Example C above and filling into a two-part hard gelatin capsule. Formulation B (*infra*) is prepared in a similar manner.

Formulation B

	<u>mg/capsule</u>
15 (a) Active ingredient	250
(b) Lactose B.P.	143
(c) Sodium Starch Glycolate	25
(d) Magnesium Stearate	2
20	420

Formulation C

	<u>mg/capsule</u>
25 (a) Active ingredient	250
(b) Macrogol 4000 BP	350
	600

Capsules are prepared by melting the Macrogol 4000 BP, dispersing the active ingredient in the melt and filling the melt into a two-part hard gelatin capsule.

5 Formulation D

	<u>mg/capsule</u>
Active ingredient	250
Lecithin	100
Arachis Oil	100
10	450

Capsules are prepared by dispersing the active ingredient in the lecithin and arachis oil and filling the dispersion into soft, elastic gelatin capsules.

15

Formulation E (Controlled Release Capsule)

The following controlled release capsule formulation is prepared by extruding ingredients a, b, and c using an extruder, followed by spheronisation of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin capsule.

	<u>mg/capsule</u>
(a) Active ingredient	250
25 (b) Microcrystalline Cellulose	125
(c) Lactose BP	125
(d) Ethyl Cellulose	13

Example E: Injectable Formulation

<u>Active ingredient</u>	0.200 g
5 Sterile, pyrogen free phosphate buffer (pH7.0) to 10 ml	

The active ingredient is dissolved in most of the phosphate buffer (35-40°C), then made up to volume and filtered through a sterile micropore filter into a sterile 10 ml amber glass vial (type 1) and sealed with sterile closures and overseals.

Example F: Intramuscular injection

Active ingredient	0.20 g
15 Benzyl Alcohol	0.10 g
Glucofurool 75°	1.45 g
Water for Injection q.s. to	3.00 ml

The active ingredient is dissolved in the glycofurool. The benzyl alcohol is then added and dissolved, and water added to 3 ml. The mixture is then filtered through a sterile micropore filter and sealed in sterile 3 ml glass vials (type 1).

Example G: Syrup Suspension

Active ingredient	0.2500 g
Sorbitol Solution	1.5000 g
5 Glycerol	2.0000 g
Dispersible Cellulose	0.0750 g
Sodium Benzoate	0.0050 g
Flavour, Peach 17.42.3169	0.0125 ml
Purified Water q.s. to	5.0000 ml

10

The sodium benzoate is dissolved in a portion of the purified water and the sorbitol solution added. The active ingredient is added and dispersed. In the glycerol is dispersed the thickener (dispersible cellulose). The two dispersions are mixed and made up to the required volume with the purified water. Further thickening is achieved as required by extra shearing of the suspension.

Example H: Suppositorymg/suppository

20 Active ingredient (63 µm)*	250
Hard Fat, BP (Witepsol H15 - Dynamit Nobel)	1770

2020

25 *The active ingredient is used as a powder wherein at least 90% of the particles are of 63 µm diameter or less.

One fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45°C maximum. The active ingredient is sifted through a 200 µm sieve and

added to the molten base with mixing, using a silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at 45°C, the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250 µm stainless steel screen and, with continuous stirring, is allowed to cool to 40°C. At a temperature of 38°C to 40°C 2.02 g of the mixture is filled into suitable plastic moulds. The suppositories are allowed to cool to room temperature.

10 Example I: Pessaries

	<u>mg/pessary</u>
Active ingredient	250
Anhydrate Dextrose	380
Potato Starch	363
15 Magnesium Stearate	7
	1000

The above ingredients are mixed directly and pessaries prepared by direct
20 compression of the resulting mixture.

28. *Use in medicine*

The aforementioned β-form or an aggregate thereof or a binding agent
25 including antibodies and other agents eg compounds of the invention or a formulation thereof may be administered in a variety of ways, for non-limiting example, by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time, depending on the

characteristics of the patient and/or the particular prion disease against which the treatment is directed.

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CLAIMS

1. A method of making a β -form of a prion protein which has more β -sheet than α -helix structure, can exist as a monomer and can retain solubility in an aqueous solution in the absence of a denaturant, the method comprising: providing a reduced form of a prion protein which does not include a disulphide bond and causing the conformation of the protein to change so that it adopts the β -form.
- 10 2. A method as claimed in Claim 1 wherein the β -form is the dominant prion protein species in the absence of a denaturant.
- 15 3. A method as claimed in Claim 1 or 2 wherein the prion protein having a β -form can retain solubility without denaturant to a concentration of more than 1mg/ml.
- 20 4. A method as claimed in Claim 3 wherein the β -form can retain solubility without denaturant to a concentration of at least 12mg/ml, and preferably more than 20mg/ml.
5. A method as claimed in any one of Claims 1 to 4 wherein the change in conformation is caused by exposure to conditions of acidic pH.
- 25 6. A method as claimed in Claim 5 wherein the pH is 4.8 or less, preferably 4.0.
7. A method as claimed in any preceding claim wherein the reduced form is denatured prior to causing the conformation to change.

8. A method of obtaining a non-aggregated β -form of a prion protein from a sample comprising partially digesting the sample with proteinase K.
9. A β -form of a prion protein which has more β -sheet than α -helix structure, can exist as a monomer and can retain solubility in aqueous solution in the absence of a denaturant.
5
10. A β -form of a prion protein which is obtainable by a method as claimed in any one of Claims 1 to 8.

10

11. A β -form of a prion protein which is non-aggregated, but capable of forming an aggregated fibrous and/or amyloid form.
12. A β -form as claimed in Claim 11 wherein the β -form is capable of forming an aggregated fibrous and/or amyloid form on exposure to a denaturant.
15
13. A β -form of a prion protein which is non-aggregated but, on exposure to conditions of sufficient ionic strength is capable of forming an aggregated non-fibrillar structure.
20
14. A β -form of a prion protein wherein the β -form is non-aggregated and exhibits partial resistance to digestion with proteinase K.
- 25 15. A preparation of a β -form of a prion protein wherein at least 1% has more β -sheet than α -helix structure, can exist as a monomer and can retain solubility in an aqueous solution in the absence of a denaturant.

16. A β -form of a prion protein as claimed in any preceding claim wherein the β -form will interconvert between a predominantly α -helical form and the said predominantly β -sheet form when in aqueous solution at a concentration of more than 1mg/ml and in the absence of a denaturant.

5

17. A protein as claimed in Claim 16 wherein the said concentration is at least 12mg/ml.

18. Use of a β -form of a prion protein as claimed in any preceding claim, or of a preparation as claimed in Claim 15, in the manufacture of a composition for use in the prevention, treatment and/or diagnosis of a prion disease.

19. A method of making an antibody against a β -form of a prion protein comprising administering said β -form or an aggregate thereof to a mammal and collecting and purifying the directly or indirectly resulting antibody.

20. A method as claimed in Claim 19 wherein the antibody is polyclonal.

20 21. A method of making an antibody against a β -form of a prion protein or an aggregate thereof the method comprising immunising a mammal with the β -form or aggregate, fusing an antibody-producing cell from the mammal with an immortal cell to form a hybridoma, and purifying a monoclonal antibody therefrom.

25

22. A monoclonal antibody capable of distinguishing between the α -form and the β -form of a prion protein.

23. A hybridoma cell capable of producing a monoclonal antibody as claimed in Claim 22.
24. A β -form binding agent for use in medicine, which agent binds 5 preferentially to the β -form of a prion protein rather than to the α -form of the prion protein.
25. Use of a binding agent as claimed in Claim 24 in the manufacture of a composition for use in the prevention, treatment and/or diagnosis of a 10 prion disease.
26. A method of making a prion protein aggregate comprising providing a β -form as defined in any preceding claim and exposing the β -form to conditions of ionic strength sufficient to cause formation of a non- 15 fibrillar aggregate.
27. A method as claimed in Claim 26 wherein the conditions of sufficient ionic strength is a salt concentration of from 50 to 500 mM.
28. A method as claimed in Claim 26 or 27 wherein the β -form is exposed to the conditions of sufficient ionic strength for from 0 to 60 mins.
29. A method as claimed in any of Claims 26 to 28 wherein the aggregate is in the form of spherical or irregularly shaped particles which can be identified by electron microscopy.
30. A method as claimed in Claim 29 wherein the particles have a diameter of from approximately 10 – 20 nm.

31. A method as claimed in any one of Claims 26 to 30 wherein the aggregate is capable of forming a fibrillar structure.

5 32. A non-fibrillar aggregate obtainable by a method as claimed in any one of Claims 26 to 31.

33. A method of making an antibody against a non-fibrillar aggregate obtainable by a method as claimed in any one of Claims 26 to 31
10 comprising administering said aggregate to a mammal; and collecting and optionally purifying the resulting antibody.

34. A method as claimed in Claim 33 wherein the antibody is polyclonal.

15 35. A method of making an antibody against a non-fibrillar aggregate obtainable by a method as claimed in any one of Claims 26 to 31 comprising immunising a mammal with the aggregate, fusing an antibody-producing cell from the mammal with an immortal cell to form a
20 hybridoma and collecting a monoclonal antibody therefrom.

36. A method as claimed in any one of Claims 19 to 23 and 33 to 35 wherein the mammal does not express a prion protein.

25 37. An antibody obtainable by a method as claimed in any one of Claims 33 to 36.

38. A hybridoma cell obtainable by a method as claimed in Claim 35 or 36 which is capable of secreting an antibody.

39. A binding agent which binds selectively to a non-fibrillar aggregate as defined in any one of Claims 26 to 32.

5 40. A binding agent as claimed in Claim 39 which binds preferentially to the non-fibrillar aggregate as defined in any one of Claims 26 to 32 rather than an aggregated fibrillar structure.

10 41. Use of a non-fibrillar aggregate obtainable by a method as claimed in any one of claims 26 to 32 in medicine.

42. Use of an aggregate as claimed in Claim 41 for the prevention, treatment and/or diagnosis of a prion disease.

15 43. A method of detecting in a biological sample the presence of a non-fibrillar aggregate as defined in any preceding claim the method comprising providing a non-fibrillar aggregate binding agent preparation comprising an agent which selectively binds the non-fibrillar aggregate and detecting whether the agent binds the aggregate in the sample.

20 44. A method of detecting antibodies in a biological sample, which antibodies bind preferentially to a non-fibrillar aggregate as defined in any preceding claim rather than the β -form and/or fibrillar form comprising exposing the non-fibrillar aggregate to the biological sample to permit binding of antibody to the aggregate and detecting the binding of antibody to the aggregate.

45. A method of obtaining a non-fibrillar aggregate binding agent which binds preferentially to a non-fibrillar aggregate of a prion protein rather than

a β -form and/or a fibrillar form comprising exposing the aggregate to a sample to permit binding of agents to the aggregate and optionally collecting the agent bound to the aggregate.

5 46. A method as claimed in Claim 45 wherein the binding agent is directly or indirectly labelled and its binding to the aggregate is detected by detecting the label.

10 47. A method as claimed in Claim 45 or 46 wherein the non-fibrillar aggregate binding agent comprises an antibody or fragment thereof.

15 48. A kit for diagnosing a prion disease from a biological sample comprising a binding agent capable of preferentially binding the non-fibrillar aggregate rather than the β -form and/or fibrillar form, or a non-fibrillar aggregate of a prion protein which binds said agent, optionally, the agent or non-fibrillar aggregate being coupled to an inert support; and means for detecting binding of the agent to the aggregate.

20 49. A kit as claimed in claim 48 wherein the means for detecting binding comprises a radioactive, enzymic or fluorescent label.

25 50. A method of identifying an agent which is capable of preventing, reducing and/or reversing the conversion of a prion protein to a β -form, the method comprising: providing a sample of a prion protein and comparing the amount of the β -form quantitatively or qualitatively in the presence and absence of a test agent.

51. A method of identifying an agent which is capable of inhibiting or reducing the conversion from a β -form of a prion protein to an aggregated

fibrous and/or amyloid form, the method comprising providing a β -form of the prion protein and comparing qualitatively or quantitatively the amount of the aggregated and/or amyloid form in the presence and absence of a test agent.

5

52. A method of identifying an agent which is capable of inhibiting or reducing the conversion from a β -form of a prion protein to an aggregate fibrous and/or amyloid, especially a non-fibrillar aggregate form, the method comprising providing a β -form of the prion protein and comparing 10 qualitatively or quantitatively the amount of the aggregated and/or amyloid, especially a non-fibrillar aggregate form in the presence and absence of a test agent.

53. A method as claimed in Claim 52 wherein the β -form is exposed to 15 conditions of ionic strength of 50 mM or more, preferably 50 to 500 mM.

54. A method as claimed in Claim 53 wherein the aggregated form is a non-fibrillar aggregate as defined in any one of Claims 26 to 42.

20 55. A method as claimed in any one of Claims 51 to 54 wherein the amount of the aggregated and/or amyloid, especially a non-fibrillar aggregate form of the prion protein is measured using a spectrofluorimeter.

56. An agent identifiable by a method as claimed in Claims 50 to 55.

25

57. An agent capable of preventing, reducing and/or reversing the conversion from a β -form of a prion protein to an aggregated and/or amyloid form.

58. An agent as claimed in Claim 57 wherein the aggregated form is a non-fibrillar aggregate as defined in any one of Claims 26 to 42.

59. An agent for treating a prion disease comprising a β -form binding agent portion which binds preferentially to the β -form of a prion protein rather than the α -form and an effector portion which is capable of one or more of the following functions: (1) preventing, reducing and/or reversing the conversion of a prion protein to a β -form; (2) preventing or reducing the conversion of a prion protein from the β -form to an aggregated fibrous and/or amyloid form, especially a non-fibrillar aggregate; or (3) destroying a β -form of a prion protein and/or a cell or virus displaying such a protein.

60. An agent as claimed in claim 59 wherein the binding agent comprises an antibody or a fragment thereof.

61. An agent as claimed in any one of Claims 56 to 60 for use in medicine.

20 62. Use of an agent as claimed in Claim 61 in the manufacture of a composition for use in the prevention, treatment and/or diagnosis of a prion disease.

25 63. A pharmaceutical composition comprising a pharmaceutically effective amount of an agent as claimed in any one of Claims 50 to 61 together with a pharmaceutically acceptable diluent or carrier.

64. A method of preventing and/or treating a prion disease comprising administering to a subject an effective amount of an agent as claimed in any one of Claims 50 to 61.

5 65. Use of a β -form of a prion protein or a non-fibrillar aggregate thereof as claimed in any preceding claim in the manufacture of a composition for use as a vaccine against a prion disease.

10 66. A vaccine composition comprising a β -form of a prion protein or a non-fibrillar aggregate thereof as claimed in any preceding claim.

67. A vaccine composition as claimed in Claim 66 further comprising an adjuvant.

15 68. An *in vitro* method for diagnosing a predisposition to, or the presence of a prion disease comprising providing a test solution containing a reduced α -form of a prion protein which has more α -helix than β -sheet, and comparing the amount or rate of formation of a β -form, as defined in any preceding claim, in the presence and absence of a sample.

20 69. A method of diagnosing a predisposition to, or the presence of, a prion disease comprising providing a β -form of a prion protein as defined in any preceding claim; providing a sample; and exposing the β -form to the sample and detecting the presence of an aggregation of the β -form, such an aggregation being indicative of predisposition to, or the presence of, a prion disease.

70. A method of treating a biological sample to remove a β -form of a prion protein or a non-fibrillar aggregate thereof comprising providing a binding agent which binds preferentially to the β -form of a prion protein rather than to the α -form of the prion protein, or a binding agent which binds preferentially to the non-fibrillar rather than the β -form and/or fibrillar aggregate; exposing the biological sample to the binding agent whereby the β -form or non-fibrillar aggregate thereof can bind the binding agent and, optionally, collecting the treated biological sample.

5

10 71. A method as claimed in claim 70 wherein the binding agent is an antibody or a fragment thereof.

72. A method as claimed in any preceding claim wherein the biological sample comprises a bodily fluid or tissue, such as whole blood, a component of blood, cerebrospinal fluid, faeces, urine, sputum, lymph, tonsil, lymph node and appendix.

15

20 73. A method of detecting in a biological sample the presence of a prion protein having a β -form, the method comprising providing a β -form binding agent preparation comprising an agent which selectively binds the β -form and detecting whether the agent binds the β -form in the sample.

74. A method of detecting antibodies in a biological sample, which antibodies bind preferentially to a β -form of a prion protein rather than the α -form comprising exposing the β -form to the biological sample to permit binding of antibody to the β -form and detecting the binding of antibody to the β -form.

25

75. A method of obtaining a β -form binding agent which binds preferentially to a β -form of a prion protein rather than an α -form comprising exposing the β -form to a sample to permit binding of agents to the β -form and optionally collecting the agent bound to the β -form.

5

76. A method as claimed in Claim 75 wherein the binding agent is directly or indirectly labelled and its binding to the β -form is detected by detecting the label.

10 77. A method as claimed in Claim 75 or 76 wherein the binding agent comprises an antibody or fragment thereof.

15 78. A kit for diagnosing a prion disease from a biological sample comprising a β -form binding agent capable of preferentially binding the β -form rather than the α -form, or a β -form of a prion protein which binds said agent, optionally, the agent or β -form being coupled to an inert support; and means for detecting binding of the agent to the β -form.

20 79. A kit as claimed in claim 78 wherein the means for detecting binding comprises a radioactive, enzymic or fluorescent label.

80. An agent capable of preventing, reducing and/or reversing the conversion of a prion protein from an α -form to a β -form.

25 81. A method of detecting a β -form of prion protein or an aggregate thereof in a sample, the method involving pre-treating the sample with proteinase k or a binding agent, such as an antibody, which binds preferentially to the cellular α -form of a prion protein, PrP^C, rather than

the β -form or an aggregate thereof; exposing the sample to a binding agent, such as an antibody, capable of binding the β -form or an aggregate thereof; and detecting binding of the binding agent to the β -form or an aggregate thereof.

5

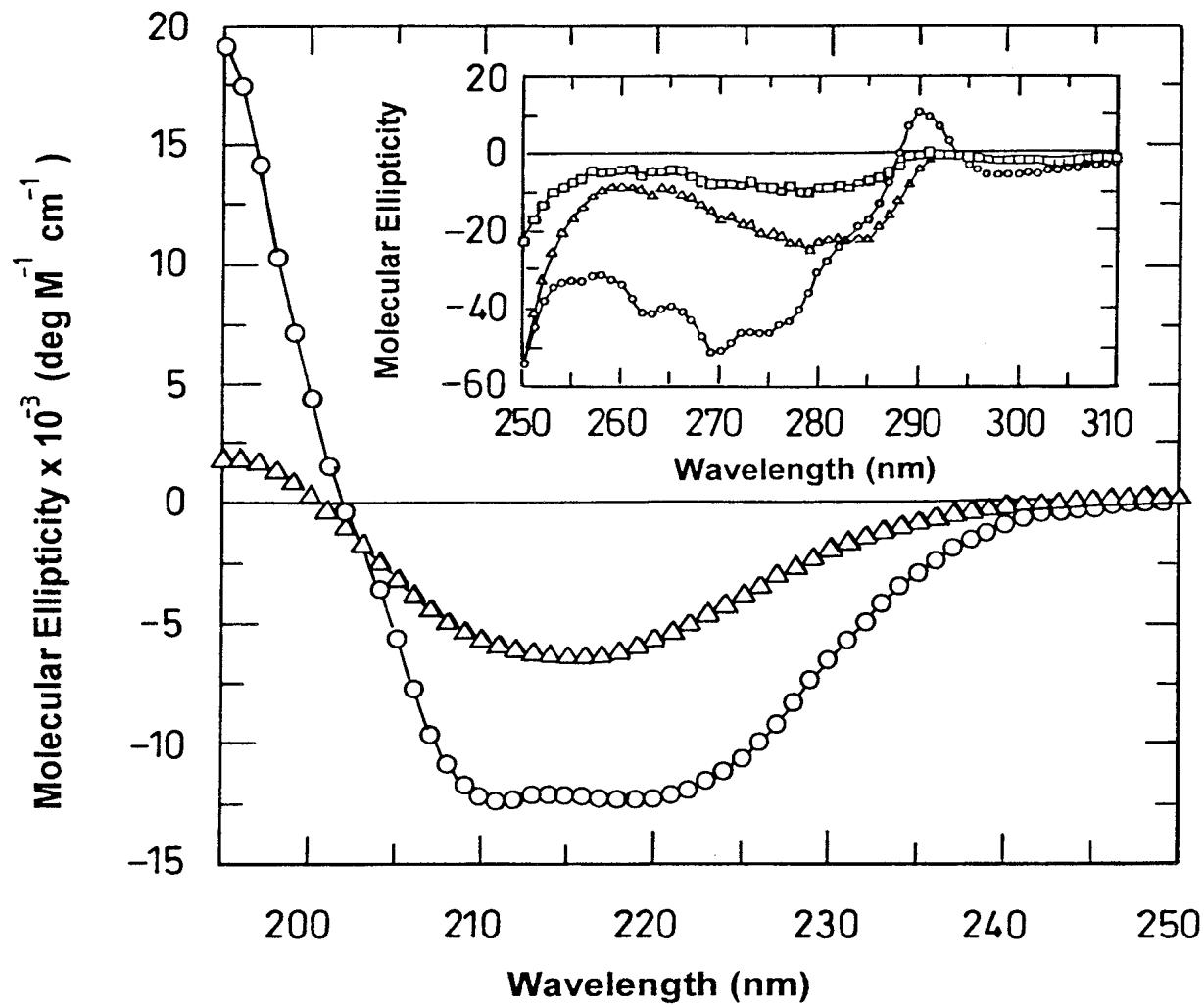
82. A method of making a β -form of a prion protein or a non-fibrillar aggregate thereof, substantially as described herein, preferably with reference to one or more of the exemplary methods.

10 83. A β -form of a prion protein or a non-fibrillar aggregate substantially as described herein, preferably with reference to one or more of the figures and exemplary methods.

15 84. A β -form of a prion protein or a non-fibrillar aggregate thereof for use substantially as described herein, preferably with reference to one or more of the exemplary methods and figures.

20 85. A method of making or using a binding agent, preferably an antibody, which binds preferentially to the β -form of a prion protein rather than the α -form, or binds preferentially to a non-fibrillar aggregate of the β -form rather than the β -form and/or fibrillar form, substantially as described herein, preferably with reference to one or more of the exemplary methods and figures.

25 86. Any novel subject matter described herein.

1/10***Fig. 1a***

2/10

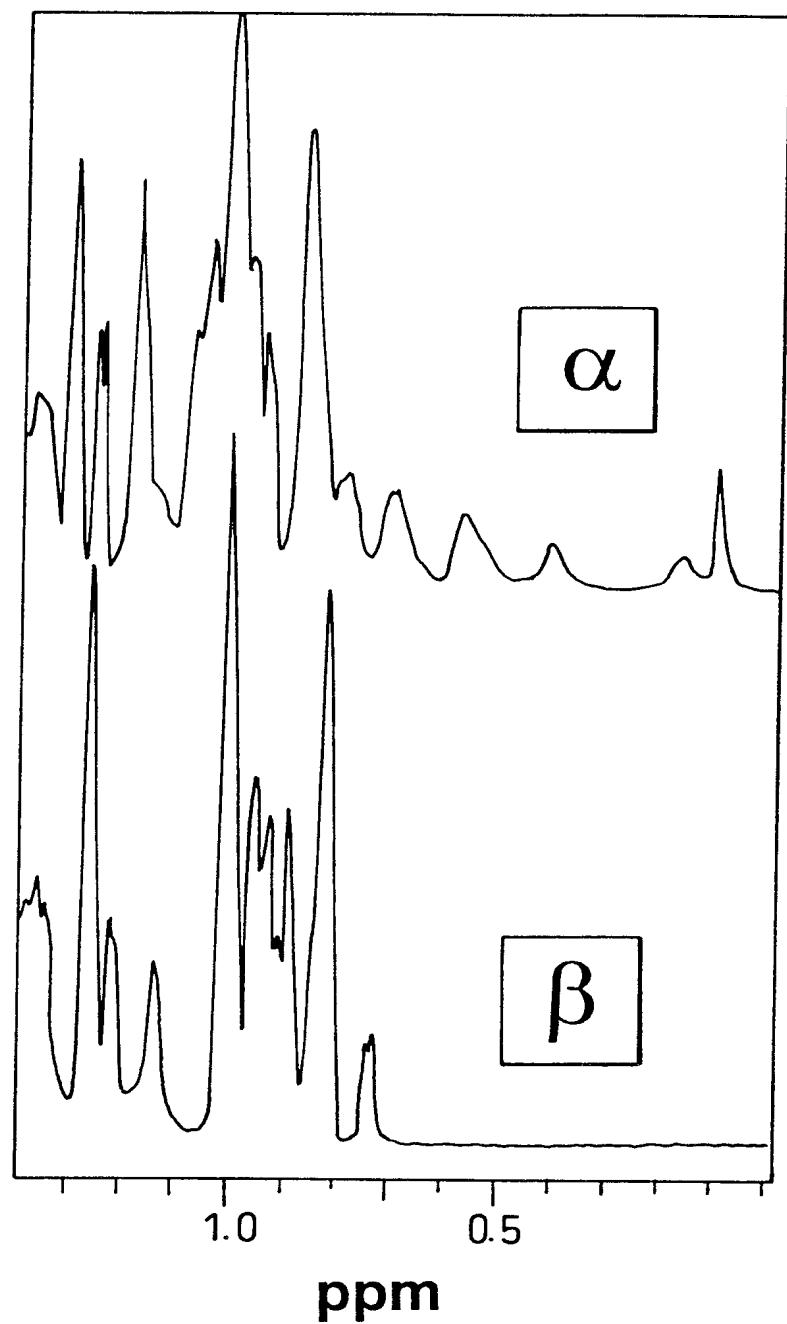


Fig. 1b

3/10

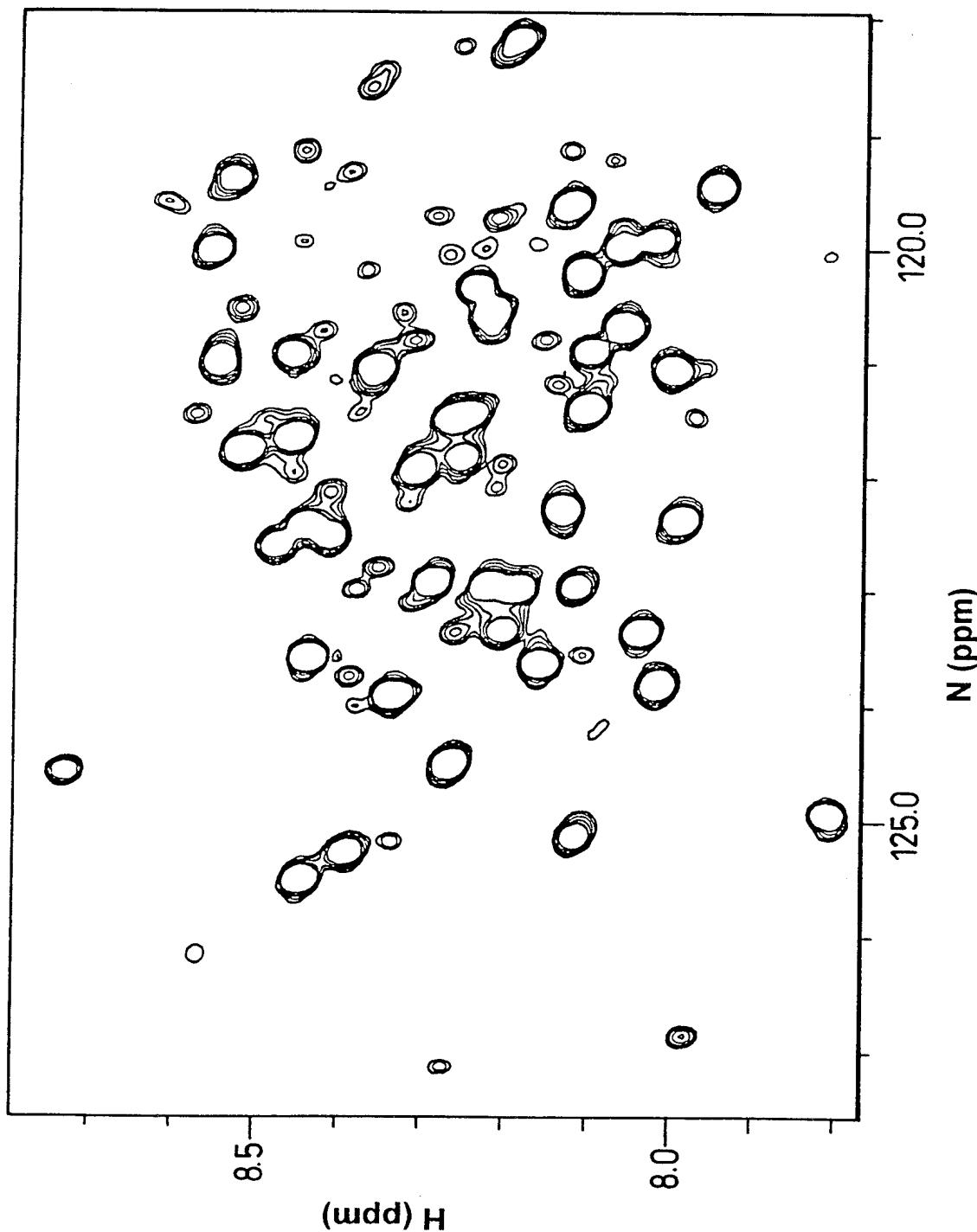


Fig. 1c

4/10

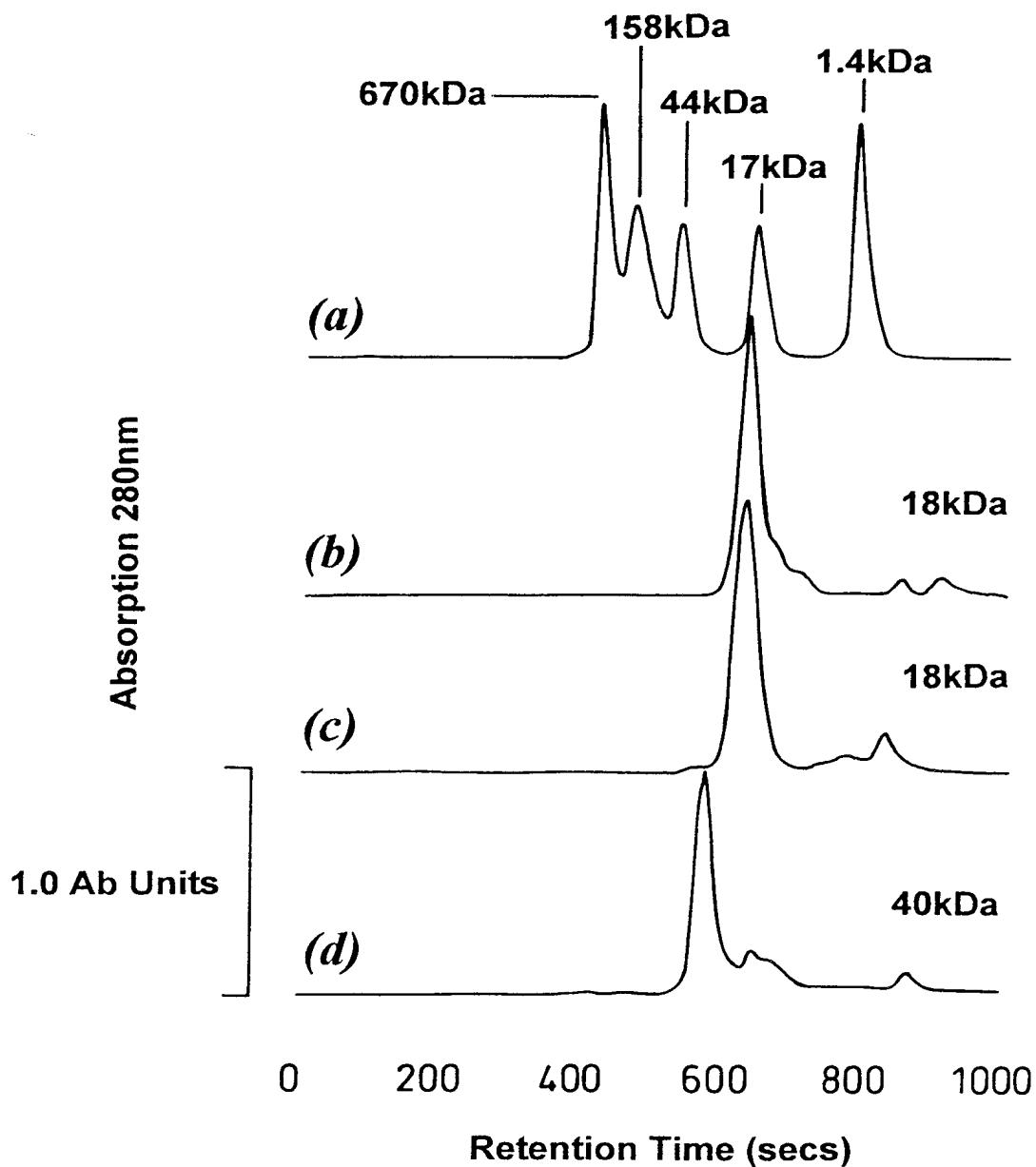


Fig. 2

5/10

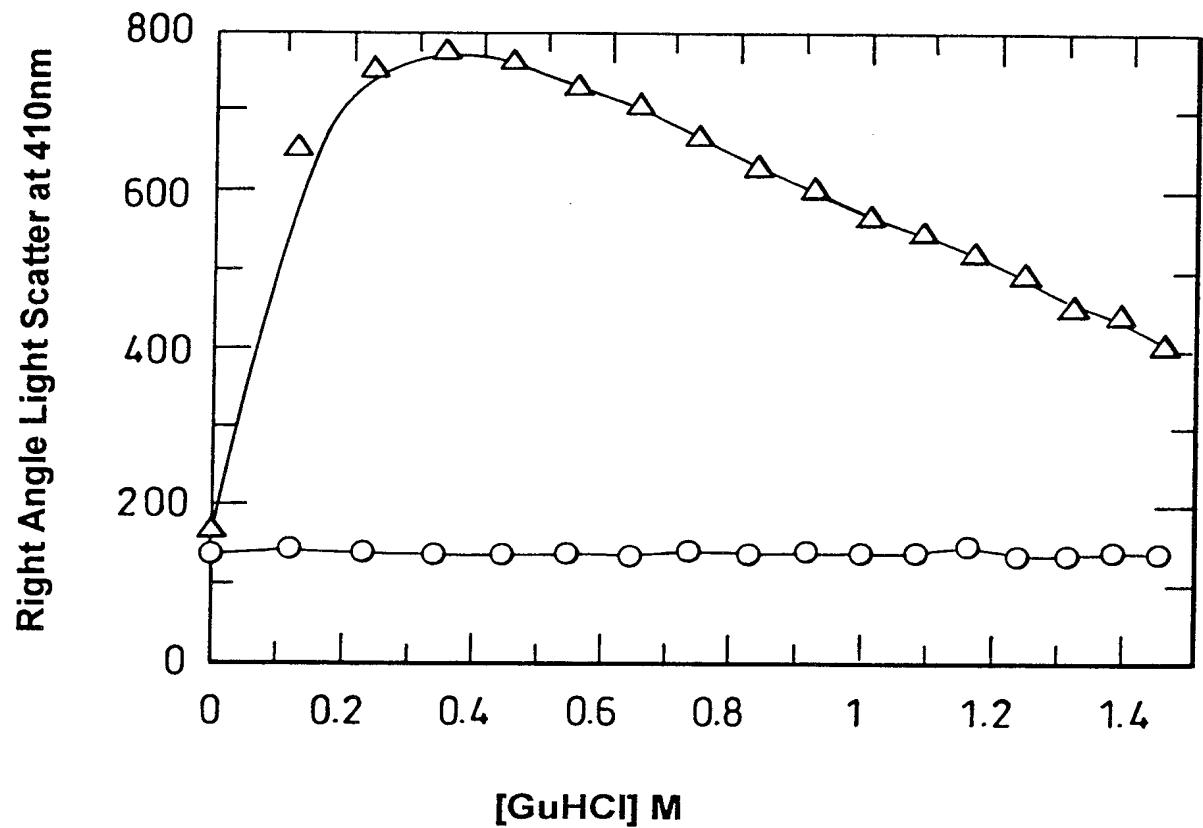


Fig. 3

6/10

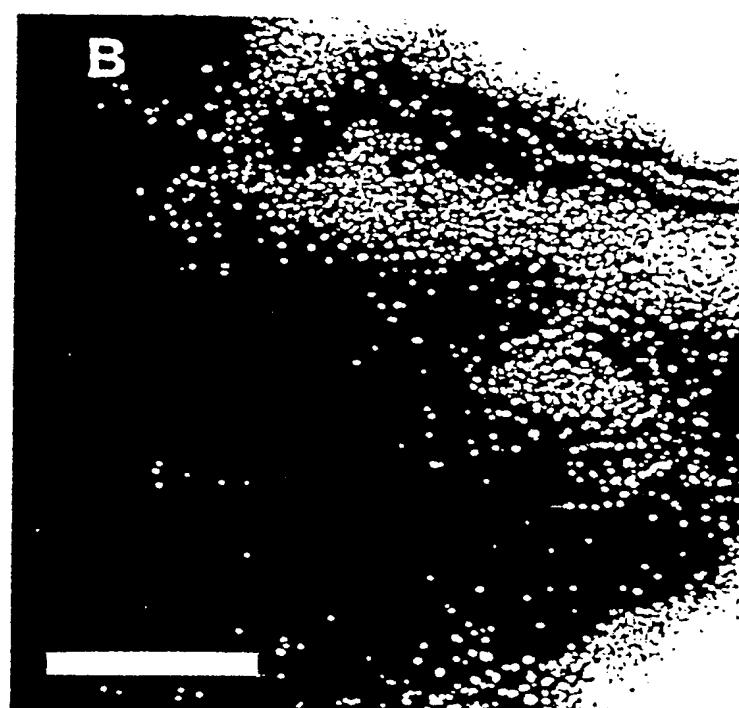
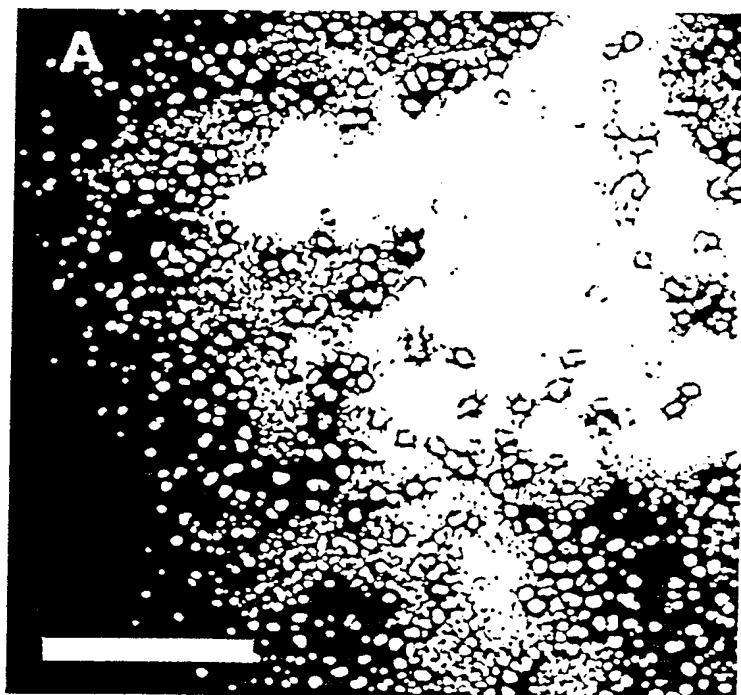


Fig. 4

7/10

PK $\mu\text{g/ml}$
0 50 5 0.05 0.005

α - PrP

— — —

Soluble

β - PrP

— — —

Aggregated

β - PrP

— — —

Fig. 5

8/10

	-----MANILGCWMLVLFVATWSDLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Human	-----MANILGCWMLVLFVATWSDLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Chimpanzee	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Orangutan	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Gorilla	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Monkey (Gr)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Monkey (S)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Rhesus	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Gibbon	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Macaque (S)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Macaque (C)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Macaque (J)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Macaque (P)	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Marmoset	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Hamadryas	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Cercopithecus	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Guereza	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Capuchin	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNLYPPQ-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Francoisi	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Siamang	-----MANILGCWMLVLFVATWSNLIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGKGOPHGGGG--WGQPHGGGWGQPHGGGW-----
Mouse (RML)	-----MANILGYWMLLVLFVATWDIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQ-----	-----GGTWGOPHGGGG--WGQPHGGSWGQPHGGSW-----
Mouse (Sh)	-----MANILGYWMLLVLFVATWDIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQ-----	-----GGTWGOPHGGGG--WGQPHGGSWGQPHGGSW-----
Mouse (Ig)	-----MANILGYWMLLVLFVATWDIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQ-----	-----GGTWGOPHGGGG--WGQPHGGSWGQPHGGSW-----
Hamster (C)	-----MANILSYWMLLVLFVATWDIGLCKK-RPKPGG-WNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGTWGOPHGGGG--WGQPHGGSWGQPHGGSW-----
Cow	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Sheep	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Antelope	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Kudu	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Goat	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Pig	-----MVKSHIGGWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Polecat	-----MVKSHIGSWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Dog	-----MVKSHIGGWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Rabbit	-----MVKSHIGGWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Marsupial	-----MVKSHIGGWMLLVLFVATWDIGLCKK-RPKPGGWNNTTGGS-RYPGQ-GSPGGNRYPPQG-----	-----GGGWGQPHGGGG--WGQPHGGGWGQPHGGGW-----
Chicken	-----MVKAMARLLTTCCLLALLLAACIDVALSKKGKGPSSGGWWAGSHRQPSYPROPG-----	-----PGSSSW-----

Fig. 6

9/10

Fig. 6 (*continued*)

10/10

Human	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG---	260
Chimpanzee	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Orangutan	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Gorilla	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Monkey (Gr)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Monkey (S)	:	NVTIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	252
Rhesus	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	267
Gibbon	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Macaque (S)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Macaque (C)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Macaque (J)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Macaque (P)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Marmoset	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Hamadryas	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	259
Cercopithe:	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Guereza	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	252
Capuchin	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Francoisi	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	259
Siamang	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGSSMVLFSSPPVILL-ISFLIFLVG*	260
Mouse (RML)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYYDGRRSSSTVLFSSPPVILL-ISFLIFLVG*	260
Mouse (Sh)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYYDGRRSSSTVLFSSPPVILL-ISFLIFLVG*	261
Mouse (Lg)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYYDGRRSSSTVLFSSPPVILL-ISFLIFLVG*	261
Hamster (C)	:	NITIKQHTV-TTTKGENFTETDVKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	261
Cow	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	261
Sheep	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	269
Antelope	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	256
Kudu	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--EAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	261
Goat	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	269
Pig	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORGASVILFSSSSTVLFSSPPVILL-ISFLIFLVG*	261
Polecat	:	NITVKQHTV-TTTKGENFTETDMKIMMERVEQMCITQYERES--EAYY--ORGASAILFSSSPPVILL-ISLLILLIVG*	262
Dog	:	NITVKQHTV-TTTKGENFTETDMKIMMERVEQMCITQYERES--EAYY--ORGASAILFSSSPPVILL-ISLLILLIVG*	262
Rabbit	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--QAYY--ORAAGVILFSSSSPPVILL-ISFLIFLVG*	261
Marsupial	:	NITVKQHTV-TTTKGENFTETDIKIMMERVEQMCITQYERES--EA--AAQRAYNMAFFSAPPVTLFLSFLFLIVS--	259
Chicken	:	NITVTEYSIGPAAKNTSEAVAAANOTEVEMENKVUTTKVIRECMCQQYREYRLASGIQJHPADTWLAVLLLTLFAMH--	264
			277

Fig. 6 (continued)